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(54) Title: VACCINES AGAINST DISEASES CAUSED BY ENTEROPATHOGENIC ORGANISMS USING ANTIGENS ENCAPSULATED WITHIN BIODEGRADABLE-BIOCOMPATIBLE MICROSPHERES

(57) Abstract

This invention is directed to oral parenteral and intestinal vaccines and their use against diseases caused by enteropathogenic organisms using antigens encapsulated within biodegradable-biocompatible microspheres.

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VACCINES AGAINST DISEASES CAUSED BY ENTEROPATHOGENIC ORGANISMS USING ANTIGENS ENCAPSULATED WITHIN BIODEGRADABLE-BIOCOMPATIBLE MICROSPHERES

I. GOVERNMENT INTEREST

The invention described herein may be manufactured, licensed and used by or for govermental purposes without the payment of any royalties to us thereon.

II. CROSS REFERENCE

This application is a continuation-in-part of U.S. Patent Application Serial No. 07/867,301 filed April 10, 1992 which in turn is a continuation in part of U.S. Patent Applicaiton Serial No. 07/805,721 which in turn is a continuation-in-part of U.S. Patent Application Serial No. 07/690,485 filed April 27, 1991, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 07/521,945 filed May 11, 1990, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 07/493,597 filed March 15, 1990, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 06/590,308, filed March 16, 1984.

III. FIELD OF THE INVENTION

This invention relates to parenteral and oral-intestinal vaccines against diseases caused by enteropathogenic organisms using antigens encapsulated within biodegradable-biocompatible microspheres (matrix).

PHASE I

IV. BACKGROUND OF THE INVENTION

Most infectious agents have their first contact with the host at a mucosal surface; therefore, mucosal protective immune mechanisms are of primary importance in preventing these agents from colonizing or penetrating the mucosal surface. Numerous studies have demonstrated that a protective mucosal immune response can best be initiated by introduction of the antigen at the mucosal surface, and parenteral immunization is not an effective method to induce mucosal immunity. Antigen taken up by the gut-associated lymphoid tissue (GALT), primarily by the Peyer's patches in mice, stimulates T helper cell (T_H) to assist in IgA B cell responses or stimulates T suppressor cells (T_s) to mediate the unresponsiveness of oral tolerance. Particulate antigen appears to shift the response towards the (T_H) whereas soluble antigens favor a response by the (T_s). Although

studies have demonstrated that oral immunization does induce an intestinal mucosal immune response, large doses of antigen are usually required to achieve sufficient local concentrations in the Peyer's patches. Unprotected protein antigens may be degraded or may complex with secretory IgA in the intestinal lumen.

One possible approach to overcoming these problems is to homogeneously disperse the antigen of interest within the polymeric matrix of appropriately sized biodegradable, biocompatible microspheres that are specifically taken up by GALT. Eldridge et. al. have used a murine model to show that orally-administered 1-10 micrometer microspheres consisting of polymerized lactide and glycolide, (the same materials used in resorbable sutures), were readily taken up into Peyer's patches, and the 1-5 micrometer size were rapidly phagocytized by macrophages. Microspheres that were 5-10 micrometers (microns) remained in the Peyer's patch for up to 35 days, whereas those less than 5 micrometer disseminated to the mesenteric lymph node (MLN) and spleen within migrating MAC-1^+ cells. Moreover, the levels of specific serum and secretory antibody to staphylococcal enterotoxin B toxoid and inactivated influenza A virus were enhanced and remained elevated longer in animals

which were immunized orally with microencapsulated antigen as compared to animals which recieved equal doses of non-encapsulated antigen. These data indicate that microencapsulation of an antigen given orally may enhance the mucosal immune response against enteric pathogens. AF/R1 pili mediate the species-specific binding of E. coli RDEC-1 with mucosal glycoproteins in the small intestine of rabbits and are therefore an important virulence factor. Although AF/R1 pili are not essential for E. coli RDEC-1 to produce enteropathogenic disease, expression of AF/R1 promotes a more severe disease. Anti-AF/R1 antibodies have been shown to inhibit the attachment of RDEC-1 to the intestinal mucosa and prevent RDEC-1 disease in rabbits. The amino acid sequence of the AF/R1 pilin subunit has recently been determined, but specific antigenic determinants within AF/R1 have not been identified.

Recent advances in the understanding of B cell and T cell epitopes have improved the ability to select probably linear epitopes from the amino acid sequence using theoretical criteria. B cell epitopes are often composed of a string of hydrophilic amino acids with a high flexibility index and a high probability of turns within the peptide structure. Prediction of T cell epitopes are based on the Rothbard

method which identifies common sequence patterns that are common to known T cell epitopes or the method of Berzofsky and others which uses a correlation between algorithms predicting amphipathic helices and T cell epitopes.

In the current study we have used these theortical criteria to predict probable T or B cell epitopes from the amino acid sequence of AF/R1. Four different 16 amino acid peptides that include the predicted epitopes have been synthesized: AF/R1 40-55 as a B cell epitope, 79-94 as a T cell epitope, 108-123 as a T and B cell epitope, and AF/R1 40-47/79-86 as a hybrid of the first eight amino acids from the predicted B cell epitope and the T cell epitope. We have used these peptides as well as the native protein to stimulate the in vitro proliferation of lymphocytes taken from the Peyer's patch, MLN, and spleen of rabbits which have recieved intraduodenal priming with microencapsulated or non-encapsulatled AF/R1. Our results demonstrate the microencapsulation of AF/R1 potentiates the cellular immune response at the level of the Peyer's patch, thus enhancing in vitro lymphocyte proliferation to both the native protein and its linear peptide antigens. CFA/I pili, rigid thread-like structures which are composed of repeating pilin subunits of 147 amino acid found on serogroups

015, 025, 078, and 0128 of enterotoxigenic E. coli (ETEC) [1-4, 18]. CFA/I promotes mannose resistant attachment to human brush borders [5]; therefore, a vaccine that established immunity against this protein may prevent the attachment to host tissues and subsequent disease. In addition, because the CFA/I subunit shares N-terminal amino acid sequence homology with CS1, CFA/II (CS2) and CFA/IV (CS4) [4], a subunit vaccine which contained epitopes from this area of the molecule may protect against infection with various ETEC.

Until recently, experiments to identify these epitopes were time consuming and costly; however, technology is now available which allows one to simultaneously identify all the T cell and B cell epitopes in the protein of interest. Multiple Peptide synthesis (Pepscan) is a technique for the simultaneous synthesis of hundreds of peptides on polyethylene rods [6]. We have used this method to synthesize all the 140 possible overlapping actapeptides of the CFA/I protein. The peptides, still on the rods, can be used directly in ELISA assays to map B call epitopes [6, 12-14]. We have also synthesized all the 138 possible overlapping decapeptides of the CFA/I protein. For analysis of T cell epitopes, these peptides can be cleaved from the rods and used in proliferation assays

[15]. Thus this technology allows efficient mapping and localization of both B cell and T cell epitopes to a resolution of a single amino acid [16]. These studies were designed to identify antigenic epitopes of ETEC which may be employed in the construction of an effective subunit vaccine.

CFA/I pili consist of repeating pilin protein subunits found on several serogroups of enterotoxigenic E. coli (ETEC) which promote attachment to human intestinal mucosa. We wished to identify areas within the CFA/I molecule that contain imunodominant T cell eptiopes that are capable of stimulating the cell-mediated portion of the immune response in primates as well as immunodominant B cell epitopes. To do this, we (a) resolved the discrepancy in the literature on the complete amino acid sequence of CFA/I, (b) immunized three Rhesus monkeys with multiple i.m. injections of purified CFA/I subunit in Freund's adjuvant, (c) synthesized 138 overlapping decapeptides which represented the entire CFA/I protein using the Pepscan technique (Cambridge Research Biochemicals), (d) tested each of the peptides for their ability to stimulate the spleen cells from the immunized monkeys in a proliferative assay (e) synthesized 140 overlapping octapeptides which respresented the entire CFA/I protein, and (f) tested

serum from each monkey for its ability to recognize the octapeptides in a modified ELISA assay. A total of 39 different CFA/I decapeptides supported a significant proliferative response with the majority of the responses occurring within distinct regions of the protein (peptides beginning with residues 8-40, 70-80, and 127-137). Nineteen of the responsive peptides contained a serine residue at positions 2, 3, or 4 in the peptide, and a nine contained a serine specifically at position 3. Most were predicted to be configured as an alpha holix and have a high amphipathic index. Eight B cell epitopes were identified at positions 3-11, 11-21, 22-29, 32-40, 38-45, 66-74, 93-101, and 124-136. The epitope at position 11-21 was strongly recognized by all three individual monkeys, while the epitopes at 93-101, 124-136, 66-74, and 22-29 were recognized by two of the three monkeys.

V. SUMMARY OF THE INVENTION

This invention relates to a novel pharmaceutical compositon, a microcapsule/sphere formulation, which comprises an antigen encapsulated within a biodegradable polymeric matrix such as poly (DL-lactide-co-glycolide) (DL-PLG), wherein the relative ratio between the lactide and glycolide component of the DL-PLG is within the range of 40:60 to 0:100, and its use, as a vaccine, in the effective

pretreatment of animals (including humans) to prevent intestinal infections caused by a virus or bacteria. In the practice of this invention, applicants found that the AF/R1 adherence factor is a plasmid encoded pilus composed of repeating pilin protein subunits that allows E. coli RDEC-1 to attach to rabbit intestinal brush borders. To identify an approach that enhances the immunogenicity of antigens that contact the intestinal mucosa, applicants investigated the effect of homogeneously dispersing AF/R1 pili within biodegradable microspheres that included a size range selected for Peyer's Patch localization. New Zealand White rabbits were primed twice with 50 micrograms of either microencapsulated or nonencapsulated AF/R1 by endoscopic intraduodenal inoculation. Lymphoid tissues were removed and cellular proliferative responses to AF/R1 and synthetic AF/R1 peptides were measured in vitro. The synthetic peptides represented possible T and/or B cell epitopes which were selected from the AF/R1 subunit sequence using theoretical criteria. In rabbits which had received nonencapsulated AF/R1, Peyer's Patch cells demonstrated slight but significnt proliferation in vitro in response to AF/R1 pili but not the AF/R1 synthetic peptides. In rabbits which had recieved microencapsulated AF/R1, Peyer's Patch cells demonstrated a markedly enhanced response to AF/R1 and

the synthetic peptides. Cells from the spleen and mesenteric lymph nodes responded similarly to AF/R1 pili in both groups of animals, while there was a greater response to the synthetic peptide AF/R1 40-55 in rabbits that had received microencapsulated AF/R1. These data demonstrate that microencapsulation of AF/R1 potentiates the mucosal cellular immune response to both the native protein and its linear peptide antigens.

VI. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the size destribution of microspheres wherein the particle size distibution (%) is (a) By number 1-5 (91) and 6-10 (9) and (b) By weight 1-5 (28) and 6-10 (72).

Figure 2 shows a scanning electron micrograph of microspheres.

Figures 3(a) and (b) show the In vitro immunization of spleen cells and demonstrates that AF/RI pilus protein remains immunogenic to rabbit spleen cells immunized in vitro after microencapsulation. AF/R1 pilus protein has been found to be immunogenic for rabbit spleen mononuclear cells in vitro producing a primary IgM antibody response specific to AF/RI. Immunization with antigen encapsulated in biodegradable, biocompatible microspheres consisting of lactide/glycolide copolymers

has been shown to endow substantially enhanced immunity over immunization with the free antigen. To determine if microencapsulated AF/RI maintains the immunogenicity of the free pilus protein, a primary in vitro immunization assay was conducted. Rabbit spleen mononuclear cells at a concentration of $3x10^5$ cells/well. Triplicate wells of cells were immunized with free AF/RI in a dose range from 15 to 150 ng/ml or with equivalent doses of AF/RI contained in microspheres. Supernatants were harvested on days 7, 9, 12, and 14 of culture and were assayed for free AF/RI pilus protein specific IgM antibody by the ELISA. Supernatant control values were subtracted from those of the immunized cells. Cells immunized with free pilus protein showed a significant positive IgM response on all four days of harvest, with the antibody response increasing on day 9, decreasing on day 12, and increasing again on day 14. Cells immunized with microencapsulated pilus protein showed a comparable positive IgM antibody response as cells immunized with free pilus protein. In conclusion, AF/RI maintains immunogenicity to rabbit spleen cells immunized in vitro after microencapsulation.

Figures 4(a) and (b) show in vitro immunization of Peyer's patch cells. Here the AF/RI pilus protein remains immunogenic to rabbit Peyer's

patch cells immunized in vitro after microencapsulation. AF/RI pilus protein has been found to be immunogenic for rabbit Peyer's patch mononuclear cells in vitro producing a primary IgM antibody response specific to AF/RI. Immunization with antigen encapsulated in biodegradable, biocompatible microspheres consisting of lactide/glycolide copolymers has been shown to endow substantially enhanced immunity over immunization with the free antigen. To determine if microencapsulated AF/RI maintains the immunogencity of the free pilus protein, a primary in vitro immunization assay was conducted. Rabbit Peyer's patch mononuclear cells at a concentration of $3x10^6$ cells/ml were cultured in 96-well, round bottom microculture plates at a final concentration of $6x10^5$ cells/well. Triplicate wells of cells were immunized with free AF/RI in a dose range from 15 to 150 ng/ml or with equivalent dose of AF/RI contained in microspheres. Supernatants were harvested on days 7, 9, 12, and 14 of culture and were assayed for free AF/RI pilus protein specific IgM antibody by the ELISA. Supernatant control values were subtracted from those of the immunized cells. Cells immunized with free pilus protein showed a significant positive IgM response on all four days of harvest, with the highest antibody response on day 12 with the highest antigen dose.

Cells immunized with encapsulated pilus protein showed a positive response on day 12 with all three antigen doses. In conclusion, AF/RI pilus protein maintains immunogenicity to rabbit Peyer's patch cells immunized in vitro after microencapsulation.

Figure 5 shows proliferative responses to AF/RI by rabbit Peyer's patch cells. Naive rabbits were primed twice with 50 micrograms of either non-encapsulated (rabbits 132 and 133) or microencapsulated (rabbits 134 and 135) AF/RI pili by endoscopic intraduodenal inoculation seven days apart. Seven days following the second priming, Peyer's patch cells were cultured with AF/RI in 96-well plates for four days followed by a terminal six hour pulse with [^{3}H]thymidine. Data shown is the SI calculated from the mean cpm of quadruplicate cultures. Responses were significant for all rabbits: 132 ($p=0.013$), 133 ($p=.0006$), 134 ($p=0.0016$), and 135 ($p=0.0026$). Responses were significantly different between the two groups. Comparison of the best responder in the nonencapsulated antigen group (rabbit 133) with the lowest responder in the microencapsulated antigen group (rabbit 134) demonstrated an enhanced response when the immunizing antigen was microencapsulated ($p=0.0034$).

Additionally, Figure 5 relates to the in vitro lymphocyte proliferation after sensitization of

rabbit lymphoid tissues with encapsulated or non-encapsulated AF/RI pilus adhesion of E. coli strain RDEC-1. The AF/RI adherence factor is a plasmid encoded pilus protein that allows RDEC-1 to attach to rabbit intestinal brush borders. We investigated the immunopotentiating effect of encapsulating purified AF/RI into biodegradable non-reactive microspheres composed of polymerized lactide and glycolide, materials used in resorbable sutures. The microspheres had a size range of 5-10 microns, a size selected for Peyer's Patch localizaiton, and contained 0.62% protein by weight. NZW rabbits were immunized twice with 50 micrograms of either encapsulated or non-encapsulated AF/RI by intraduodenal later of non-encapsulated AF/RI by intraduodenal inoculation seven days apart. Lymphocyte proliferation in respone to purified AR/RI was conducted in vitro at seven days and showed that encapsulating the antigen into microspheres enhanced the cellular immune response in the Peyer's Patch; however, no significant increase was observed in spleen or mesenteric lymph node. These data suggest that encapsulation of AF/RI may potentiate the mucosal cellular immune response.

Figures 6 a-d show proliferative responses to AF/RI synthetic peptides by rabbit Peyer's patch cells. Naive rabbits were primed twice with 50

micrograms of either non-encapsulated (rabbits 132 and 133) or microencapsulated (rabbits 134 and 135) AF/RI pili by endoscopic intraduodenal inoculation seven days apart. Seven days following the second priming, Peyer's patch cells from each rabbit were cultured with AF/R1 40-55 (Fig. 6a), AF/R1 79-94 (Fig. 6b), AF/R1 108-123 (Fig 6c), or AF/R1 40-47/79-86 (Fig. 6d) in 96-well plates for four days followed by a terminal six hour pulse with [^{3}H]thymidine. Data shown is the SI calculated from the mean cpm of quadruplicate cultures. The responses of rabbits 132 and 133 were not significant to any of the peptides tested. Rabbit 134 had a significant response to (a) AF/R1 40-55 ($p=0.0001$), (b) AF/R1 79-94 ($p=0.0280$), and (d) AF/R1 40-57/79-86 ($p=0.025$), but not to (c) AF/R1 108-123. Rabbit 135 had a significant response to (a) AF/R1 40-55 ($p=0.034$), (b) AF/R1 79-94 ($p=0.040$), and (c) AF/R1 108-123 ($p<0.0001$), but not to (d) AF/R1 40-47/79-86. This demonstrates enhanced proliferative response to peptide antigens following mucosal priming with microencapsulated pili. AF/RI pili promotes RDEC-1 attachment to rabbit intestinal brush borders. Three 16 amino acid peptides were selected by theoretical criteria from the AF/RI sequence as probable T or B cell epitopes and were synthesized: AF/RI 40-55 as a B cell epitope, 79-94 as a T cell

epitope, and 108-123 as a T and B cell epitope. We used these peptides to investigate a possible immunopotentiating effect of encapsulating purified Af/RI pili into biodegradable, biocompatible microspheres composed of polymerized lactide and glycolide at a size range that promotes localization in the Peyer's Patch (5-10 micrometers). NZW rabbits were primed twice with 50 micrograms AF/RI by endoscopic intraduadenal inoculation and their Peyer's Patch cells were cultured in vitro with the AF/RI peptides. In two rabbits which had received encapsulated AF/RI, lymphocyte proliferation was observed to AF/RI 40-55 and 79-94 in both rabbits and to 108-123 in one of two rabbits. No responses to any of the peptides were observed in rabbits which received non-encapsulated AF/RI. These data suggest that encapsulation of AF/RI may enhance the cellular response to peptide antigens.

Figures 7a-d show B-cell responses of Peyer's patch cells to AF/R1 and peptides.

Figures 8a-d show B-cell responses of Peyer's Patch cells to AF/R1 and peptides.

Figures 9a-d show B-cell responses of spleen cells to AF/R1 and Peptides.

Figures 10a-d show B cell responses of spleen cells to AF/R1 and peptides.

Figures 7 through 10, illustrate enhanced lymphocyte antibody response by mucosal immunization of rabbits with microencapsulated AF/R1 pilus protein. The AF/RI pilus protein has been found to be immunogenic for rabbit spleen and Peyer's patch cells in vitro producing a primary IgM antibody response. The purpose of this study was to determine if AR/R1 pilus protein immune response is enhanced by microencapsulation. The AF/R1 was incorporated into biodegradable, biocompatible microspheres composed of lactide-glycolide copolymers, had a size range of 5-10 micrometer and containing 0.62% pilus protein by weight. Initially, NZW rabbits were immunized twice with 50 micrograms of either encapsulated or non-encapsulated AF/RI via intraduodenal route seven days apart. For in vitro challenge, $6x10^5$ rabbit lymphocytes, were set in microculture at final volume of 0.2 ml. Cells were challenged with AR/RI or three different synthetic 16 amino acid peptides representing, either predicted T, B or T and B cell epitopes in a dose range of 15 to 150 ng/ml for splenic cells or 0.05 to 5.0 micrograms/ml for Peyer's patch mononuclear cells (in triplicate). Supernatants were collected on culture days 3, 5, 7, and 9 assayed by ELISA for anti-AF/R1 antibody response as compared to cell supernatant control. Significant antibody

responses were seen only from spleen and Peyer's patch cells from rabbits immunized with microencapsulated AF/R1. The antibody response tended to peak between days 5 and 9 was mainly an IgM response. The results for the predicted epitopes were similar to those obtained with purified AF/RI. In conclusion, intestinal immunization with AF/RI pilus protein contained within microspheres greatly enhances both the spleen and Peyer's patch B-cell responses to predicted T & B-cell epitopes.

Figure 11 shows proliferative responses to AF/R1 40-55 by rabbit MLN cells. Naive rabbits were primed twice with 50 micrograms of either nonencapsulated (rabbits 132 and 133) or microencapsulated (rabbits 134 and 135) AF/R1 pili by endoscopic intraduodenal inoculation seven days apart. Seven days following the second priming, MLN cells were cultured with AF/R1 40-55 for four days in 24-well plates. Cultures were transferred into 96-well plates for a terminal [^{3}H]thymidine pulse. Data shown is the SI calculated from the mean cpm of quadruplicate cultures. Responses of rabbits 132 and 133 were not statistically significant. Responses were significant for rabbits 134 ($p=0.0.0051$) and 135 ($p=0.0055$).

Figure 12 shows proliferative responses to AF/R1 40-55 by rabbit spleen cells. Naive rabbits were

primed twice with 50 micrograms of either nonencapsulated (rabbits 132 and 133) or microencapsulated (rabbits 134 and 135) AF/R1 pili by endoscopic intraduodenal inoculation seven days apart. Seven days following the second priming, spleen cells were cultured with AF/R1 40-55 for four days in 24-well plates. Cultures were transferred into 96 well plates for a terminal [^{3}H]thymidine pulse. Data shown is the SI calculated from the mean cpm of quadruplicate cultures. Responses of rabbits 132 and 133 were not statistically significant. Responses were significant for rabbits 134 (p=0.0.0005) and 135 (p=0.0066).

Figure 16. A. SDS-PAGE of intact CFA/I (lane 1), trypsin treated CFA/I (lane 2), and S. aureus V8 protease treated CFA/I. Molecular masses of individual bands were estimated from molecular weight standards (on left). Multiple lanes of both trypsin and V8 treated CFA/I were transferred to PVDF membranes where bands corresponding to the approximate molecular masses of 3500 (trypsin digest, see arrow lane 2) and 6000 (V8 digest, see arrow lane 3) were excised and subjected to Edman degradation. B. Resulsting sequence of protein fragments from each lane of A (position of sequenced portion of fragment in the intact protein. Underlined, italisized residues are amino acids under dispute in literature.

Figure 17. ELISA assay results testing hyperimmune sera of monkeys (A)2Z2 (monkey 3), (B) 184(D) (monkey 1) and (C) 34 (monkey 2) to CFA/I primary structure immobilized on polyethylene pins. Monkey sera diluted 1:1000. Peptide number refers first amino acid in sequence of octapeptide on pin from CFA/I primary structure OD 405 refers to optical density wavelength at which ELISA plates were reat (405 nm).

Figure 18. Complete sequence of CFA/I (147 amno acids) with B cell recognition site (boxed areas) as defined by each individual monkey response (2Z2, 184D, and 34). Derived from data in Figure 17.

Figures 19-21. Lymphocyte proliferation to synthetic decapeptides of CFA/I. Each monkey was immunized with three i.m. injections of CFA/I subunits in adjuvant, and its spleen cells were cultured with synthetic decapeptides which had been constructed using the Pepscan technique. The decapeptides represented the entire CFA/I protein. Concentrations of synthetic peptide used included 6.0, 0.6, and 0.06 micrograms/ml. Values shown represent the maximum proliferative response produced by any of the three concentrations of antigen used ± the standard deviation. The cpm of the control peptide for each of the three monkeys was 1,518 ± 50, 931 ± 28, and 1,553 ± 33 respectively. The cpm

of the media control for each of the three monkeys was 1,319 ± 60, 325 ± 13, and 1,951 ± 245 respectively.

Figures 22-24. Lymphocyte proliferation to 6.0, 0.6, and 0.06 micrograms/ml synthetic decapeptides of CFA/I in one monkey. The monkey (2Z2) as immunized with three i.m. injections of CFA/I subunits in adjuvant, and its spleen cells were cultured with synthetic decapeptides which had been constructed using the Pepscan technique. The decapeptides represented the entire CFA/I protein. Values shown represent the proliferative response which occurred to 6.0 micrograms/ml (Fig. 22), 0.6 micrograms/ml (Fig. 23), or 0.06 micrograms/ml (Fig. 24) of antigen ± the standard deviation. The cpm of the control peptide was 1,553 ± 33 and the cpm of the media control was 1,951 ± 245.

Figure 25 shows that rabbits numbers 21 and 22 received intraduodual administration of AF/R1 microspheres at doses of AF/R1 of 200 micrograms (ug) on day 0 and 100 ug on day 7, 14, and 21 then sacrificed on day 31. The spleen, Peyer's patch and ileal lamina propria cells at 6 x 10^5 in 0.2 ml in quadriplate were challenged with AF/RI and AF/R1 1-13, 40-55, 79-94, 108-123, and 40-47, 79-85 synthetic peptides at 15, 1.5 and .15 ug/ml for 4 days. The supernatants were tested for IL-4 using the IL-4/IL-2

dependent cell line cells CT4R at 50,000/well with 0.1 ml of 6.25% supernatant for 3 days then pulsed with tritiated thymidine for 4 hrs, cells harvested and the tritiated thymidine incorporation determined, averaged and expressed with one standard deviation thousand counts per minute (kcpm).

Figure 26 shows that RDEC-1 colonization (log CFU/gm) in cecal fluids was similar in both groups (mean 6.3 vs 7.3; p=.09).

Figure 27 shows that rabbits given AF/R1-MS remained well and 4/6 gained weight after challenge, whereas 9/9 unvaccinated rabbits lost weight after challenge (mean weight change +10 vs -270 grams p<.001).

Figure 28 shows that the mean score of RDEC-1 attachment to the cecal epithelium was zero in vaccinated, and 2+ in unvaccinated animals.

Figure 29. Particle size distribution of CFA/II microsphere vaccine Lot L74F2 values are percent frequency of number or volume verses distribution. Particle size (diameter) in microns. 63% by volume are between 5-10 um and 88% by volume are less then 10 um.

Figure 30. Scanning electron photomicrograph of CFA/II microsphere vaccine Lot L7472 standard bar represents 5 um distance.

Figure 31. Twenty-two hour CFA/II release study of CFA/II microsphere vaccine Lot L7472. Percent cumulative release of CFA/II from three sample: A, 33.12 mgm; B, 29.50 mgm c, 24.20 mgm at 1, 3, 6, 8, 12 and 22 hour intervals. Average represents the mean ± ISD.

Figure 32. Serum IgG antibody reponse to CFA/II microsphere vaccine Lot L7472 following 2 25 ug protein IM immunization on day 0 in 2 rabbits. Antibody determines on serial dilution of sera by ELISA and expressed as mean titer versus day 0, 7 and 14.

Figure 33. Serum IgG antibody response to CFA/II microsphere vaccine Lot L7F2 following 2 25 ug protein IM immunizations on day 0 if rabbit 107 & 109. Antibody determined on serial dilution (in duplicate) of sera by ELISA and expressed as mean titer versus day 0, 7 and 14.

Figure 34. Lymphocyte proliferative responses for Peyer's patch cells of rabbits 65 (figure 34 (a)), 66 (figure 34 (b)) , 83 (figure 34 (c)), 86 (figure 34 (d)), and 87 (figure 34 (e)) immunized intraduodenally with 50 mgm protein of CFA/II microsphere vaccine 4 and 7 days earlier. The cells are challenged <u>in vitro</u> with CFA/II or BSA at 500, 50 and 5 ug/ml or media in triplicate. The uptake of tritiated thymidine in Kcp is expressed as mean ± ISD.

Using the paired student t-test, the p values of 500 ug/ml dose of CFA/II compared to media control are: 65,p = 0.0002; 66,p = 0.0002; 83,p = 0.0002; and 86, p = 0.0002.

Figure 35. Lymphocyte proliferative responses from Peyer's patch cells of rabbits 77 (figure 35 (a)), 78 (figure 35 (b)), 80 (figure 35 (c)), 88 (figure 35 (d)), and 91 (figure 35 (e)) immunized introduodenally with 50 mgm protein of CFA/II microspheres vaccine 14 and 7 days earlier. The cells are challenged in vitro with CFA with CFA/II or BSA at 500, 50 and 5 ug/ml or media in triplicate the uptake of triciplate. The uptake of tritiated thymidine in Kcp is expressed as mean ± ISD. Using the paired student t -test, the protein of 500 ug/ml dose of CFA/II compared to media control are: 77, p = 0.0001; 78; = 0.0015; 80, p = insignificant; 88, p = 0.0093; and 91 p = 0.0001.

Figure 36. ELISPOT assay of spleen cells from rabbits 65 (figure 36 (a)), 66 (figure 36 (b)), 83 (figure 36 (c)), 86 (figure 36 (d)), and 87 (figure 36 (e)) immunized intraduodenally with 50 mgm protein of CFA/II microsphere vaccine 14 and 7 days earlier. These were cells placed into microculture and tested on day 0, 1, 2, 3, 4 and 5 by ELISPOT for cells secreting antibodies specific for CFA/II antigen. The

results are expressed as number per 9 x 10^6 spleen cells versus culture day tested.

Figure 37. ELISPOT assay of spleen cells from normal control rabbits, 67, 69, 72 and 89. The cells were placed into microculture and tested on days 0, 1, 2, 3, 4 and 5 by ELISPOT for cells secreting antibodies specific for CFA/II antigen. The results are expressed as number per 9 x 10^6 spleen cells versus culture day tested.

Figure 38. Curve for determining vaccination dosages for regimen b.

Figure 39. Hepatitis B surface antigen release from 50:50 poly (DL-lactide-co-glycolide).

Figures 11 and 12 serve to illustrate that inclusion of Escherichia coli pilus antigen in microspheres enhances cellular immunogenicity.

A primary mucosal immune response, characterized by antipilus IgA, follows infection of rabbits with E. coli RDEC-1. However, induction of an optimal primary mucosal response by enteral vaccination with pilus antigen depends on immunogenicity of pilus protein, as well as such factors as its ability to survive gastrointestinal tract (GI) transit and to target immunoresponsive tissue. We tested the effect of incorporating AF/R1 pilus antigen into resorbable microspheres upon its ability to induce primary mucosal

and systemic antibody responses after direct inoculation into the GI tract. METHODS: rabbits were inoculated with 50 micrograms of AF/R1 pilus antigen alone or incorporated into uniformaly sized (5-10 microns) resorbably microspheres (MIC) of poly(DL-lactide-coglycolide). Inoculation was by intra-duodenal (ID) intubation via endoscopy or directly into the ileum near a Peyer's patch via the RITARD procedure (with the cecum ligated to enhance recovery of gut secretions and a reversible ileal tie to slow antigen clearance). ID rabbits were sacrificed at 2 weeks for collection of gut washes and serum. RITARD rabbits were bled and purged weekly for 3 weeks with Co-lyte to obtain gut secretions. Anti-pilus IgA and IgG were measured by ELISA.

TABLE 1

RESULTS:*pos/test RITARD-PILI RITARD-MIC ID-PILI ID-MIC

Anti-pilus IgA (fluid)	*7/8	4/8	1/2	0/3
Anti-pilus IgG (serum)	0/8	3/8	0/2	1/3

Native pilus antigen led to a mucosal IgA resposne in 7/8 RITARD rabbits. MIC caused a similar response in only 4/8, but the groups were not statistically different. MIC (but not pili) induced

some systemic IgG responses (highest in animals without mucosal responses). Results in rabbits inoculated ID were similar for pili, but no mucosal response to ID-MIC was noted. SUMMARY: Inoculation with pilus antigen produces a primary mucosal IgA response. Microencapsulation does not enhance this response, although the antigen remains immunogenic as shown by measurable mucosal and some strong serum responses. It must be determined whether priming with antigen in microspheres can enhance secondary responses.

B CELL EPITOPE DATA

Materials and Methods

CFA/I PURIFICATION- INTACT CFA/I pili were purified from H10407 (078:H-) as described by Hall et al, (1989) [20]. Briefly, bacteria grown on colonization factor antigen agar were subjected to shearing, with the shearate subjected to differential centrifugation and isopycnic banding on cesium chloride in the presence of N-lauryl sarkosine. CFA/I were dissociated to free subunits in 6M guanididinium HC1, 0.2 M ammonium bicarbonate (2 hr, 25°), passed through an ultrafiltration membrane (Amicon XM 50 stirred cell, Danvers, MA), with concentration and buffer exchange to PBS on a YM 10 stirred cell (Amicon). Examination of dissociated pili by electron microscopy demonstrated a lack of pilus structure.

Protein Sequencing- The primary structure of CFA/I has been determined by protein sequencing techniques (Klemm, 1982) and through molecular cloning methods (Karjalainen, et al 1989) [21]. In these two studies there was agreement in all but two of the 147 amino acid residues (at positions 53 and 74). To resolve the apparent discrepancies, CFA/I was enzymatically digested in order to obtain internal amino acid sequence. Trypsin or S. aureus V8 protease (sequencing grade, Boehringer Mannheim) was incubated with CFA/I at a 1:50 w:w ratio (Tris 50 mM, 0.1% SDS, pH 8.5 for 16h at 37° (trypsin) or 24°C (V8)). Digested material was loaded onto precast 16% tricine SDS-PAGE gels (Schagger and von Jagow, 1987) (Novex, Encinitis, CA) and run following manufacturers instructions. Separated samples were electrophoretically transferred to PVDF membranes (Westrans, Schleicher and Schuell, Keene, NH) following Matsiduria (1987) using the Novex miniblot apparatus. Blotted proteins were stained with Rapid Coomassie stain (Diversified Biotech, Newton Centre, MA). To obtain the desired fragment containing the residue of interest within a region accessible by automated gas phase sequencing techniques, molecular weights were estimated from standards of molecular weights 20,400 to 2,512 (trypsin inhibitior, myoglobin, and myoglobin cyanogen bromide fragments; Diversified

Biotech) using the corrected molecular weights for the myoglobin fragments as given in Kratzin et al., (1989) [22]. The estimated molecular weights for the unknown CFA/I fragments were compared to calculated molecular weights of fragments as predicted for CFA/I from the sequence of CFA/I as analysed by the PEPTIDESORT program of a package developed by the University of Wisconsin Genetics Computer Group. Selected fragments were cut from the PVDF emebrane and subjected to gas phase sequencing (Applied Biosystem 470, Foster City, CA).

Monkey Immunization- Three rhesus monkeys (Macaca mulatta) were injected intramuscularly with 250 ug of dissociated CFA/I in complete Freund's adjuvent and subsequently with two injections of 250 ug of antgen in incomplete Freund's adjuvent at weekly intervals. Blood was drawn three weeks after primary immunization.

Peptide Synthesis- Continuous overlapping octapeptides spanning the entire sequence CFA/I were synthesized onto polyethylene pins by the method of Geysen et al. [16], also known as the PEPSCAN procedure. Derivitized pins and software were purchased from Cambridge Research Biochemicals (Valley Stream, NY). Fmoc-amino acid pentafluorophenyl esters were purchased from Peninsular Laboratories (Belmont, CA), 1-hydroxybenzotriazole monohydrate (HYBT) was

purchased from Aldrich, and reagent grade solvents from Fisher. To span the entire sequence of CFA/I with a single amino acid overlap of from one peptide to the next, 140 total pins were necessary, with a second complete set of 140 pins synthesized simultaneously.

ELISA procedure- Sera raised in monkeys to purified dissociated pili were incubated with the pins in the capture ELISA assay of Geysen et al., [16] with the preimmune sera of the same animal tested at the same dilution simultaneously with the duplicate set of pins. Dilution of sera used on the pins was chosen by initial titration of sera by standard ELISA assay and immunodot blot assay against the same antigen.

RESULTS

It was essential to utilize the correct sequence of CFA/I in the synthesis of the pins for both T- and B-cell experiments to carry out the studies as planned. At issue were the amino acids at position 53 and 74; incorrect residues at those positions would effect 36 of 138 pins (26%) for T-cell epitope analysis and 30 of 140 pins (21%) for B-cell analysis. To resolve the discrepancy in the literature, purified CFA/I was proteolytically digested separately with trypsin and with S..aureus V8 protease (V8). These enzymes were chosen in order to give fragments with the residues of interest (53 and 74) relatively near to the N-terminus

for automated Edman degradation (preferably 1-15 residues). These digests were separated on tricine SDS-PAGE gels (Fig. 16A) and molecular masses of fragments estimated. A fragment of 3459 calculated molecular mass is expected from the trypsin digest (corresponding to amino acids 62-94) and a fragment of 5889 calculated molecular mass is expected from the V8 digest (residues 42-95). These fragments were located within each digest (arrows in Fig. 16), and a companion gel with four lanes of each digest was run, electrophoreticaly transferred to PVDF, the bands excised and sequenced. N-terminal sequences of each fragment are given in Fig. 16B. The N-terminal eighteen residues from the trypsin fragment were determined that corresponded to positions 62-79 in CFA/I. Position 74, a serine residue was consistent with that determined by Karjalainen et al., (Karjalainen et al., 1989). Nineteen residues of the V8 fragment were determined, corresponding to residues 41-60 of the parent protein. The twelfth residue of the fragment contained an aspartic acid, also consistent with Karjalainen et al., (1989). All other residues sequenced were consistent with those published previously (including residues 1-29, not shown). For the following peptide synthesis were therefore

utilized the complete amino acid sequence of CFA/I consistent with Karjalainen et al., (1989).

Sera from monkeys immunized with CFA/I subunits were tested in a modified ELISA assay, with the preimmunization sera tested simultaneously with duplicate pins. Assays results are displayed in Fig. 17. Monkey 2Z2 (fig. 2A) responded strongly to six regions of the CFA/I sequence. Peptide 14 (the octapeptide 14-21) gave the strongest response with four pins adjacent to it (11, 12, 13, and 15) also appearing to bind significant antibody. The other 2Z2 epitopes are centered at peptides 3, 22, 33, 93, and 124. Monkey 184D (Fig. 17B) also responded strongly to peptide 14, although the maximum response was to peptide 13, with strong involvement of peptide 12 in the epitope. Additional epitopes recognized by 184d were centered at peptides 22, 33, 66, and 93. The third monkey serum tested, 34, responded to this region of the CFA/I primary structure, both at peptides 1, 12 and weakly at 14. Two other epitopes were identified by 34, centered at peptides 67 and 128. Figure 18 illustrates the amino acids corresponding to the epitopes of CFA/I as defined by the response of these three monkeys aligned with the entire primary structure. The entire antigenic determinants are mapped and areas of overlap with other epitopes

(consensus sites) are displayed. These epitopes are summarized in Table 1.

T Cell Epitope Data

Materials and Methods

Animals. Three healthy adult Macaca mulatta (Rehesus) monkeys (approximately 7 kg each) were used in this study. Their medical records were examined to assure that they had not been in a previous protocol which would preclude their use in this study. Each monkey was sedated with ketamine HCL1 at standard dosage and blood was drawn to obtain preimmune serium.

Antigen. CFA/I pili were purified from E. coli strain H107407 (serotype 078:H11) by ammonium sulfate precipitation using the method of Isaacson [17]. The final preparation migrated as a single band on SD-polyacrylamide gel electrophoresis and was shown to be greater than 95% pure by scanning with laser desitometry when stained with coomassie blue. The pili were then dissociated into CFA/I pilin subunits.

Immunization. Each monkey was given 25 mg of purified CFA/I pilin subunits, which had been emulsified in Complete Freund's Adjuvant, by single i.m. injection (0.5 ml). For each animal, the initial dose of antigen was followed by two similar injections in Incomplete Freund's Adjuvant at seven day intervals.

Peptide Antigens. The peptides were synthesized based on the published sequence of CFA/I [18] using the Geysen pin method (Pepscan procedure) [16] with equipment and software purchased from Cambridge Research Biochemicals, Inc. (Wilmington, DE). Fmoc-amino acid pentafluorophenyl esters were purchased from Peninsula Laboratories (Belmont, CA) and used without further treatment or analysis. The activating agent 1-hydroxybenzotriazole monohydrate (HOBT) was purchased from Aldrich Chemical Company (Milwaukee, WI). Solvents were reagent grade from Fisher Scientific (Springfield, NJ).

Two schemes were used to synthesize the peptides. Peptides for the B-cell tests were synthesized as octamers and remained linked to the resin. However, the peptides used to search for T-cell epitopes were synthesized as decamers with an additional Asp-Pro spacer between the pins and the peptides of interest. The Asp-Pro linkage is acid labile allowing cleavage of the decamers from the pins for T-cell proliferation assays [15]. The peptides were cleaved in 70% formic acid for 72 hours at 37 degrees C. The acid solution was removed by evaporation (Speed-Vac, Savant Instruments, Framingdale, NY) followed by rehydration with distilled deionized water and lyophilizaiton. The resulting cleaved peptides were used without further

treatment or analysis. The yield was approximately 10 ug per pin, approximately 10 per cent on a molar basis of the total amount of proline on each pin as determined by quantitative amino acid analysis.

Residues 12 and 13 on the CFA-1 protein are Asp and Pro, respectively, the same sequence used to cleave the peptides from the pins. Therefore, to prevent truncated peptides from the native sequence during the cleavage process, two substitutions were made for Asp-12. One substitution was a glutamic acid residue for the aspartic acid, a substitution to retain the carboxylic acid functional group. The second substitution was an asparagine residue to conserve the approximate size of the side chain while retaining some hydrophilicity. Each substitution was tested in the T-cell proliferation assay. Both substitutions as well as the native sequence were analyzed by ELISA. For both the T cell and B cell assays, additional sequences not found on the protein were synthesied and used as control peptides.

Lymphocyte proliferation. At day 10-14 following the final inoculation of antigen, the monkeys were again sedated with ketamine HCl, and 50 ml of blood was drawn from the femoral artery for serum preparation. Animals were then euthanized with an overdose of pentothal and spleen was removed. Single cell

suspensions were prepared and washed in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, NY) which had been supplemented with penicillin (100 units/ml), streptomycin (100 ug/ml), L-glutamine (2mM), and HEPES Buffer (10 mM) all obtained from Gibco Laboratories, as well as MEM non-essential amino acid solution (0.1 mM), MEM [50x] amino acids (2%), sodium bicarbonate (0.06%), and 5 x 10^{-5} M 2-ME all obtained from Sigma Chemical Company (St. Louis, MO) [cDMEM]. Erythrocytes in the spleen cell suspension were lysed using standard procedures in an ammonium chloride lysing buffer. Cell suspensions were adjusted to 10^{7} cells per ml in cDMM, and autologous serum was added to yield a final concentration of 1.0%. Cells (0.05 ml) were plated in 96-well flat bottom culture plates (Costar, Cambridge, MA) along with 0.05 ml of various dilutions of antigen in cDMEM without serum (yielding a 0.5% final concentration of autologous serum) and were incubated at 37 degrees C in 5% CO_2. Each peptide was tested at 6.0, 0.6, 0.06 ug/ml. All cultures were pulsed with 1 uci [^{3}H]thymidine (25 Ci/mmol, Amersham, Arlington Hights, IL) on day 4 of culture and were harvested for scintillation counting 6 hours later.

ELISA.

Epitope prediction. Software designed to predict B cell epitopes based on hydrophilicity, flexibility,

and other criteria was developed by the University of Wisconsin Genetics Computer Group [19]. Software designed to predict T cell epitopes based on the Rothbard method [7] was written by Stephen Van Albert (The Walter Reed Army Institute of Research, Washington, D.C.). Software designed to predict T cell epitopes based on the Berzofsky method was published as the AMPHI program [9]. It predicts amphipathic amino acid segments by evaluating 7 or 11 residues as a block and assigning the score to the middle residue of that block.

Statistics. All lymphocyte proliferations were conducted in replicates of four, and standard deviations of the counts per minute (cpm) are shown. Statistical significance (p value) for the proliferative assay was determined using the Student's t test to compare the cpm of quadruplicate wells cultured with the CFA/I peptides to the cpm of wells cultured with a control peptide.

RESULTS

Prediction of T cell epitopes within the CFA/I molecule. To identify possible T cell epitopes within the CFA/I molecule, amphipathic amino acid segments were predicted by evaluating 7 or 11 residues as a block using the AMPHI program [9]. Possible t cell epitopes were also identified using criteria published

by Rothbard and Taylor [7]. The sequence numbers of the first amino acid of the predicted segments are shown in Table 1.

Lymphocyte proliferation of monkey spleen cells to CFA/I synthetic peptides. To determine which segments of the CFA/I protein are able to stimulate proliferation of CFA/I immune primate lymphocytes in vitro, three Rhesus monkeys were immunized with CFA/I subunits, and their splenic lymphocytes were cultured with synthetic overlapping decapeptides which represented the entire CF/I sequence. Concentrations of peptides used as antigen were 6.0, 0.6, and 0.6 ug/ml. Proliferative responses to the decapeptides were observed in each of the three monkeys (fig. 1-3). The majority of the responses occurred at the 0.6 and 0.06 ug/ml concentrations of antigen and within distinct regions of the protein (peptides beginning with residues 8-40, 70-80, and 27-137). A comparison of the responses at the 6.0, 0.6 and 0.06 ug/ml concentrations antigenic peptide for one monkey (2&2) are shown (fig. 4-6). Taking into account all concentrations of antigen tested, spleen cells from monkey 184D demonstrated a statistically significant response to decapeptides beginning with CFA/I amino acid residues 3, 4, 8, 12, 15, 21, 26, 28, 33, 88, 102, 10, 133, 134, and 136 (fig. 19). Monkey 34 had a

significant response to decapeptides beginning with residues 24, 31, 40, 48, 71, 72, 77, 78, 80, 87, and 102, 126 and 133 (Fig. 20); monkey 2Z2 responded to decapeptides which began with residues 4, 9, 11, 12, 13, 14, 15, 16, 17, 20, 27, 35, 73, 79, 18, 127, 129, 132, and 133 (fig. 19). Peptides beginning with amino acid residues 3 through 2 were synthesized with either a glutamic acid or an asparagine substituted for the aspartic acid residue at position twelve to prevent truncated peptides. The observed responses to peptides beginning with residue 8 (monkey 184d), and residues 9, 11, 12 (monkey 2Z2) occurred in response to peptides that had the glutamic acid substitution. However, the observed responses to peptides beginning with residue 3, 4, and 12 (monkey 184D), a well as residue 4 (monkey 2Z2) occurred in response to peptides that had the asparagine substitution. Monkey 34 did not respond to any of the peptides that had the substitution at position twelve. All other responses shown were to the natural amino acid sequence of the CFA/I protein. Statistical significance was determined by comparing the cpm of quadruplicate wells cultured with the CFA/I peptides to the cpm of wells cultured with the CFA/I peptides to the cpm of wells cultured with a control peptide.

Analysis of decapeptides that supported proliferation of lymphocytes from CFA/I immune animals. Of the 39 different peptides that supported proliferative responses, thirty contained a serine residue, 19 contained a serine at either position 2, 3, or 4, and nine had a serine specifically at position 3. Some of the most robust responses were to the peptides that contained a serine residue at the third position. The amino acid sequence of four such peptides is shown in Table 3.

VII. DETAILED DESCRIPTION OF THE INVENTION

Applicants have discovered efficacious pharmaceutical compositions wherein the relative amounts of antigen to the polymeric matrix are within the ranges of 0.1 to 1.5% antigen (core loading) and 99.9 to 98.5% polymer, respectively. It is preferred that the relative ratio between the lactide and glycolide component of the poly(DL-lactide-co-glycolide) (DL-PLG) is within the range of 40:60 to 0:100. However, it is understood that effective core loads for certain antigens will be influenced by its microscopic form (i.e. bacteria, protozoa, viruses or fungi) and type of infection being prevented. From a biological perspective, the DL-PLG or glycolide monomer excipient are well suited for *in vitro* drug (antigen) release because they elicit a

minimal inflamatory response, are biologically compatible, and degrades under physiologic conditions to products that are nontoxic and readily metabolized.

Surprisingly, applicants have discovered an extremely effective method for the protection against bacterial or viral infections in the tissue of a mammal (human or nonhuman animal) caused by enteropathogenic organisms comprising administering orally to said animal an immunogenic amount of a pharmaceutical composition consisting essentially of an antigen encapsulated within a biodegradable polymeric matrix. When the polymeric matrix is DL-PLG, the most preferred relative ratio between the lactide and glycolide component is within the range of 48:52 to 58:42. The bacterial infection can be caused by bacteria (including any derivative thereof) which include Salmonella typhi, Shigella sonnei, Shigella flexneri, Shigella dysenteriae, Shigella boydii, Escheria coli, Vibro cholera, yersinia, staphylococcus, clostridium and campylobacter. Representative viruses contemplated within the scope of this invention, susceptible to treatment with the above-described pharmaceutical compositions, are quite extensive. For purposes of illustration, a partial listing of these viruses (including any derivative thereof) include hepatitis A, hepatitus B, rotaviruses, polio virus human

immunodeficiency viruses (HIV), Herpes Simplex virus type 1 (cold sores), Herpes Simplex virus type 2 (Herpesvirus genitalis), Varicella-zoster virus (chicken pox, shingles), Epstein-Barr virus (infectious mononucleosis; glandular fever; and Burkittis lymphoma), and cytomegalo viruses.

A further representation description of the instant invention is as follows:

A. (1) To homogeneously disperse antigens of enteropathic organisms within the polymeric matrix of biocompatible and biodegradable microspheres, 1 nanogram (ng) to 12 microns in diameter, utilizing equal molar parts of polymerized lactide and glycolide (50:50 DL-PLG, i.e. 48:52 to 58:42 DL-PLG) such that the core load is within the range of about 0.1 to 1.5% by volume. The microspheres containing the dispered antigen can then be used to immunize the intestine to produce a humoral immune response composed of secretory antibody, serum antibody and a cellular immune response consisting of specific T-cells and B-cells. The immune response is directed against the dispered antigen and will give protective immunity against the pathogenic organism from which the antigen was derived.

(2) AF/R1 pilus protein is an adherence factor that allows E. coli RDEC-1 to attach to rabbit intestinal brush borders thus promoting colonization

resulting in diarrhea. AF/R1 pilus protein was homogeneously dispered within a polymeric matrix of biocompatible and biodegradable microspheres, 1-12 microns in diameter (Figure 1 and photograph 1) using equal molar parts of polymerized lactide and glycolide (50:50 DL-PLG) such that the core load was .62% by weight.

(3) The microspheres were found to contain immunogenic AF/R1 by immunizing both rabbit spleen (Figure 2) and Peyer's patch (Figure 3) B-cells in vitro. The resultant cell supernatants contained specific IgM antibody which recognized the AF/R1. The antibody response was comparable to immunizing with AF/R1 alone.

(4) Microspheres containing 50 micrograms of AF/R1 were used to intraintestinally (intraduodenally) immunize rabbits on two separate occasions 1 week apart. One week later, compared to rabbits receiving AF/R1 alone, the intestinal lymphoid tissue, Peyer's patches, demonstrated an enhanced cellular immune response to AF/R1 and to three AF/R1 linear peptide fragments 40-55, 79-94 and 108-123 by both lymphocyte transformation (T-cells) (Figures 4 and 5) and antibody producing B-cells (Figures 6 and 7). Similarly enhanced B-cell responses were also detected in the spleen (Figures 8 and 9). An enhanced T-cell

response was also detected in the mesenteric lymph node and the spleen to one AF/R1 peptide fragment, 40-55 (Figures 10 and 11). The cellular immune response at two weeks was too early for either a serum or secretory antibody response (See Results in Table 1); but indicates that a secretory antibody response will develop such that the rabbits so immunized could be protected upon challenge with the E. coli RDEC-1.

B. Microspheres do not have to be made up just prior to use as with liposomes. Also liposomes have not been effective in rabbits for intestinal immunization of lipopolysaccharide antigens.

C. (1) Only a small amount of antigen is required (ugs) when dispersed within microspheres compared to larger amounts (mgms) when antigen is used alone for intestinal immunization.

(2) Antigen dispersed within microspheres can be used orally for intestinal immunization whereas antigen alone used orally even with gastric acid neutralization requires a large amount of antigen and may not be effective for intestinal immunization.

(3) Synthetic peptides with and without attached synthetic adjuvants representing peptide fragments of protein antigens can also be dispersed within microspheres for oral-intestinal immunization.

Free peptides would be destroyed by digestive processes at the level of the stomach and intestine. Any surviving peptide would probably not be taken up by the intestine and therefore be ineffective for intestinal immunization.

(4) Microspheres containing antigen maybe placed into gelatin-like capsules for oral administration and intestinal release for improved intestinal immunization.

(5) Microspheres promote antigen uptake from the intestine and the development of cellular immune (T-cell and B-Cell) responses to antigen components such as linear peptide fragments of protein antigens.

(6) The development of intestinal T-cell responses to antigens dispersed within microspheres indicate that T-cell immunological memory will be established leading to long-lived intestinal immunity. This long-lived intestinal immunity (T-cell) is very difficult to establish by previous means of intestinal immunization. Failure to establish long-lived intestinal immunity is a fundamental difficulty for intestinal immunizaiton with non-viable antigens. Without intestinal long-lived immunity only a short lived secretory antibody response is established

lasting a few weeks after which no significant immunological protection may remain.

D. (1) Oral intestinal immunization of rabbits against E. coli RDEC-1 infection using either whole killed organisms, pilus protein preparations or lipopolysaccharide preparations.

(2) Microspheres containing adherence pilus protein AF/R1 or its antigen peptides for oral intestinal immunization of rabbits against RDEC-1 infection.

(3) Oral-intestinal immunization of humans against enterotoxigenic E. coli infection using either whole killed organisms, pilus protein preparations or lipopolysaccharide preparations.

(4) Microspheres containing adherence pilus proteins CFA/I, II, III and IV or their antigen peptides for oral intestinal immunization of humans against human enterotoxigenic E. coli infections.

(5) Oral-intestinal immunization of humans against other enteric pathogens as salmonella, shigella, camphlobacter, hepatitis-A virus, rota virus and polio virus.

(6) Oral-intestinal immunization of animals and humans for mucosal immunological protection at distal mucosal sites as the bronchial tree in lungs, genito-urinary tract and breast tissue.

E. (1) The biocompatible, biodegradable co-polymer has a long history of being safe for use in humans since it is the same one used in resorbable suture material.

(2) By using the microspheres, we are now able to immunize the intestine of animals and man with antigens not normally immunogenic for the intestinal mucosa because they are either destroyed in the intestine, unable to be taken up by the intestinal mucosa or only weakly immunogenic if taken up.

(3) Establishing long-lived immunological memory in the intestine is now possible because T-cells are immunized using microspheres.

(4) Antigens that can be dispersed into microspheres for intestinal immunization include the following: proteins, glycoporteins, synthetic peptides, carbohydrates, synthetic polysaccharides, lipids, glycolipids, lipopolysaccharides (LPS), synthetic lipopolysaccharides and with and without attached adjuvants such as synthetic muramyl dipeptide derivatives.

(5) The subsequent immune response can be directed to either systemic (spleen and serum antibody) or local (intestine, Peyer's patch) by the size of the microspheres used for the intestinal immunization. Microspheres 5-10 microns in diameter remain within

macrophage cells at the level of the Peyer's patch in the intestine and lead to a local intestinal immune response. Microspheres 1 ng - 5 microns in diameter leave the Peyer's patch contained within macrophages and migrate to the mesenteric lymph node and to the spleen resulting in a systemic (serum antibody) immune response.

(6) Local or systemic antibody mediated adverse reactions because of preexisting antibody especially cytophyllic or IgE antibody may be minimized or eliminated by using microspheres because of their being phagocytized by macrophages and the antigen is only available as being attached to the cell surface and not free. Only the free antigen could become attached to specific IgE antibody bound to the surface of mast cells resulting in mast cell release of bioactive amines necessary for either local or systemic anaphylaxis.

(7) Immunization with microspheres containing antigen leads to primarily IgA and IgG antibody responses rather than an IgE antibody response, thus preventing subsequent adverse IgE antibody reactions upon reexposure to the antigen.

In addition to the above, the encapsulation of the following synthetic peptides are contemplated and

considered to be well within the scope of this invention:

(1) AF/R1 40-55;

(2) AF/R1 79-94;

(3) AF/R1 108-123;

(4) AF/R1 1-13;

(5) AF/R1 pepscan 16AA;

(6) CFA/I 1-13; and

(7) CFA/I pepscan 16AA.

(8) Synthetic Pepetides Containing CFA/I Pilus Protein

T-cell Epitopes (Starting Sequence # given)

4(Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro),

8(Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu),

12(Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

15(Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala),

20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

26(Pro-Ser-Ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro),

72(Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser),

78(Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln),

87(Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe),

126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr), and

133(Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val), and mixtures thereof.

(9) Synthetic Peptides Containing CFA/I Pilus Protein B-cell (antibody) Eptiopes (Starting Sequence # given)

3(Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),

11(Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

22(Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),

32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),

38(Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),

66(Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),

93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),

127(Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and mixtures thereof.

(10) synthetic peptides containing CFA/I pilus protein T-cell and B-cell (antibody) epitopes (Starting Sequence # given)

3(Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),

8(Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

11(Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and

126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and mixtures thereof.

(11) synthetic peptides containing CFA/I pilus protein T-cell and B-cell (antibody) epitopes (Starting Sequence # given)

CFA/I pilus protein T-cell epitopes

4(Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro),

8(Thr-Ala-Ser-Val-Asp-Pro-Val-Ile-Asp-Leu),

12(Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

15(Ile-Asp-Leu-Leu-Gln-Ala-Asp-Gly-Asn-Ala),

20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

26(Pro-Ser-Ala-Val-Lys-Leu-Ala-Tyr-Ser-Pro),

72(Leu-Asn-Ser-Thr-Val-Gln-Met-Pro-Ile-Ser),

78(Met-Pro-Ile-Ser-Val-Ser-Trp-Gly-Gly-Gln),

87(Gln-Val-Leu-Ser-Thr-Thr-Ala-Lys-Glu-Phe),

126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr), and

133(Gly-Asn-Tyr-Ser-Gly-Val-Val-Ser-Leu-Val); and

synthetic peptides containing CFA/I pilus protein B-cell (antibody) epitopes (Starting Sequence # given)

CFA/I pilus protein B-cell epitopes

3(Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),

11(Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

22(Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),

32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),

38(Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),

66(Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),

93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),

127(Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and mixtures thereof.

(12) synthetic peptides containing CFA/I pilus protein T-cell and B-cell (antibody) epitopes (Starting Sequence # given)

CFA/I pilus protein T-cell epitopes

3(Lys-Ana-Ile-Thr-Val-Thr-Ala-Ser-Val),

11(Val-Asp-Pro-Val-Ile-Asp-Leu-Leu-Gln-Ala-Asp),
22(Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe-Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),
32(Ala-Tyr-Ser-Pro-Ala-Ser-Lys-Thr-Phe),
38(Lys-Thr-Phe-Glu-Ser-Tyr-Arg-Val),
66(Pro-Gln-Leu-Thr-Asp-Val-Leu-Asn-Ser),
93(Ala-Lys-Glu-Phe-Glu-Ala-Ala-Ala),
124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr),
127(Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser),
and

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser); and

synthetic peptides containing CFA/I pilus protein T-cell and B-cell (antibody) epitopes (Starting Sequence # given)

<u>CFA/I pilus protein B-cell epitopes</u>

3(Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Bal-Asp-Pro),

8(Thr-Ala-Ser-Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

11(Bal-Asp-Pro-Bal-Ile-Asp-Leu-Leu-Gln-Ala-Asp),

20(Ala-Asp-Gly-Asn-Ala-Leu-Pro-Ser-Ala-Val),

124(Lys-Thr-Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and

126(Ala-Gly-Thr-Ala-Pro-Thr-Ala-Gly-Asn-Tyr-Ser), and mixtures thereof.

We contemplate that the peptides can be used in vaccine constructed for systemic administration.

VIII. EXAMPLES

The peptides in (8), (9), and (10) above can be made by classical solution phase synthesis, solid phase synthesis or recombinant DNA technology. These peptides can be incorporated in an oral vaccine to prevent infection by CFA/I bearing enteropathogenic E. coli.

The herein offered examples provide methods for illustrating, without any implied limitation, the practice of this invention in the prevention of diseases caused by enteropathogenic organisms.

The profile of the representative experiments have been chosen to illustrate the effectiveness of the immunogenic polymeric matrix-antigen composites.

All temperatures not otherwise indicated are in degrees Celcius (°C) and parts or percentages are given by weight.

IX. MATERIALS AND METHODS

Animals. New Zealand White male rabbits were purchased from Hazelton Research Products (Denver, PA), and were shown to be free of current RDEC-1 infection by culture of rectal swabs. Animals were 1-2 kg of body weight and lacked agglutinating anti-AF/R1 serum antibody at the time of the study.

Antigens. AF/R1 pili from E. coli RDEC-1 (015:H:K non-typable) were purified by an ammonium sulfate precipitation method. The final preparation migrated as a single band on SDS-polyacrylamide gel electrophoresis and was shown to be greater than 95% pure by scanning with laser densitometry when stained with coomassie blue. Briefly, equal molar parts of DL-lactide and glycolide were polymerized and then dissolved to incorporate AF/R1 into spherical particles. The microspheres contained 0.62% protein by weight and ranged in size from 1 to 12 micrometers. Both the microencapsulated and non-encapsulated AF/R1 were sterilized by gamma irradiation (0.3 megarads) before use.

Synthetic peptides (16 amino acids each) were selected by theoretical criteria from the amino acid

sequence of AF/R1 as deduced from the nucleotide sequence. Three sets of software were used for the selections. Software designed to predict B cell epitopes based on hydrophilicity, flexibility, and other criteria was developed by the University of Wisconsin Genetics Computer Group. Software designed to predict T cell epitopes was based on the Rothbard method was written by Stephen Van Albert (The Walter Reed Army Institute of Research, Washington, D.C.). Software designed to predict T cell epitopes based on the Berzofsky method is published as the AMPHI program. The selected peptides were synthesized by using conventional Merrifield solid phase technology. AF/R1 40-55 (Thr-Asn-Ala-Cly-Thr-Asp-Ile-Gly-Ala-Asn-Lys-Ser-Phe-Thr-Leu-Lys) was chosen as a probable B cell epitope for two reasons: (a) due to its high hydrophilic and flexibility indices, and (b) because it was not predicted to be a T cell epitope by either the Rothbard or Berzofsky method. AF/R1 79-94 (Val-Asn-Gly-Ile-Gly-Asn-Leu-Ser-Gly-Lys-Ala-Ile-Asp-Al a- His-Val) was selected as a probable T cell eptiope because it contained areas predicted as a T cell epitope by both methods and because of its relatively low hydrophilic and flexibility indices. AF/R1 108-123 (Asp-Thr-Asn-Ala-Asp-Lys-Glu-Ile-Lys-Ala-Gly-Gin-Asn-Thr-Val-Asp) was selected

as both a T and B cell epitope. AF/R1 40/47/79-86 was produced in continuous synthesis (Thr-Asn-Ala-Cly-Thr-Asp-Ile-Gly-Val-Asn-GlyIle-Gly-Asn-Leu-Ser) and represents a hybrid of the first eight amino acids from the predicted B cell epitope and the T cell epitope. The purity of each peptide was confirmed by C-8 reverse phase HPLC, and all peptides were desalted over a Sephadex G-10 Column before use. Using a standard ELISA method, all peptides were assayed for their ability to specifically bind anti-AF/R1 IgG antibody in hyperimmune serum from a rabbit which had received intramuscular injections of AF/R1 pili in Freund adjuvant. Only the peptide chosen as a probable B cell epitope (AF/R1 40-55) was recognized by the hyperimmune serum.

EXAMPLE 1

Immunization. Rabbits were primed twice with 50 micrograms of either microencapsulated or non-encapsulated AF/R1 by endoscopic intraduodenal inoculation seven days apart by the following technique. All animals were fasted overnight and sedated with an intramuscular injection of xylazine (10 mg) and Ketamine HCl (50 mg). An Olympus BF type P10 endoscope was advanced under direct visualization through the esophagus, stomach, and pylorus, and a 2 mm ERCP catheter was inserted through the biopsy channel

and threaded 2-3 cm into the small intestine. Inoculums of pili or pili embedded in microspheres were injected through the catheter into the duodenum and the endoscope was withdrawn. Animals were monitored daily for signs of clilnical illness, weight gain, or colonization by RDEC-1.

EXAMPLE 2

Lymphocyte Proliferation. Seven days following the second priming, the rabbits were again sedated with a mixture of xylazine and katamine HCl, and blood was drawn for serum preparation by cardiac puncture. Animals were then euthanized with an overdose of pentothal and tissues including Peyer's patches from the small bowel, MLN, and spleen were removed. Single cell suspension were prepared and washed in Dulbeco's modified Eagle medium (Gibco Laboratories, Grand Island, NY) which had been supplemented with penicillin (100 units/ml), streptomycin (100 micrograms/ml), L-glutamine (2mM), and HEPES Buffer (10 mM) all obtained from Gibco Laboratories, as well as MEM non-essential amino acid solution (0.1 mM), MEM [50x] amino acids (2%), sodium bicarbonate (0.06%), and $5x10^{-5}$ micrograms 2-ME all obtained from Sigma Chemical Company (St. Louis, MO) [cDMEM]. Erythrocytes in the spleen cell suspension were lysed using standard procedures in an ammonium chloride lysing buffer. Cell

suspension were adjusted to 5 x 10^6 cells per ml in cDMEM, and autologous serum was added to yield a final concentration of 0.5%. Cells (0.1 ml) were placed in 96-well flat bottom culture plates (Costar, Cambridge, MA) along with 0.1 ml of various dilutions of antigen and were incubated at 37°C in 5% CO_2. In other experiments, cultures were conducted in a 24-well plates. In these experiments, 5 x 10^6 cells were cultured with or without antigen in a 2 ml volume. After 4 days, 100 microliters aliquots of cells were transferred to 96-well plates for pulsing and harvesting. Previous experiments have demonstrated that optimal concentrations of antigen range from 150 ng/ml to 15 micrograms/ml in the 96-well plate assay and 1.5 ng/ml to 150 ng/ml in the 24-well plate assay. These were the concentrations employed in the current study. All cultures were pulsed with 1 Ci [^{3}H]thymidine (25 Ci/mmol, Amersham, Arlington Heights, IL) on day 4 of culture and were harvested for scintillation counting 6 hours later.

Statistics. All cultures were conducted in replicates of four, and standard deviations of the counts per minute (cpm) generally range from 5-15% of the average cpm. In experiments where comparison of individual animals and groups of animals is desirable, data is shown as a stimulation index (SI) to facilitate

the comparison. SI were calculated by dividing the mean of cultures with antigen by the mean of cultures without antigen (media control). Statistical significance (p value) was determined by comparing the maximum response for each antigen to the media control using the Student's t test.

IX. RESULTS

Lymphocyte proliferation in response to protein and peptide antigens of AF/R1. To determine if lymphoid tissues from AF/R1 immune animals respond *in vitro* to the antigens of AF/R1, the immunity in a rabbit with preexisting high levels of anti-AF/R1 serum IgG was boosted twice by injection of 50 micrograms of purified AF/R1 pili i.p. seven days apart. A week after the final boost, *in vitro* lymphocyte proliferation of spleen and MLN cells demonstrated a remarkable response to AF/R1 pili (Fig. 13). In response to the synthetic peptides, there was a small, but significant proliferation of the spleen cells to all the AF/R1 peptides tested as compared to cell cultures without antigen (Fig. 14). Cells from the spleen and Peyer's patches of non-immune animals failed to respond to either AF/R1 or the synthetic peptides.

Microencapsulation of AF/R1 potentiates the mucosal cellular immune response. To evaluate the effect that microencapsulation of AF/R1 may have on the

cellular mucosal immune response to that antigen, naive rabbits were primed twice with 50 micrograms of either microencapsulated or non-encapsulated AF/R1 by endoscopic intraduodenal inoculation seven days apart. All rabbits were monitored daily and showed no evidence of clinical illness or colonization by RDEC-1. One week following the last priming, the rabbits were sacrificed and lymphoid tissues were cultured in the presence of AF/R1 pili or peptide antigens. In rabbits which had received non-encapsulated AF/R1, Peyer's Patch cells demonstrated a low level but significant proliferation in vitro in response to AF/R1 pili (Fig. 5), but not to any of the AF/R1 synthetic peptides (Fig. 6a-6d). However, in rabbits which had received microencapsulated AF/R1, Peyer's Patch cells demonstrated a markedly enhanced response not only to AF/R1 (Fig. 5), but now responded to the AF/R1 synthetic peptides 40-55 and 79-94 (Fig. 6a and 6b). In addition, one of two rabbits primed with microencapsulated AF/R1 (rabbit 135) responded to AF/R1 108-123, but not AF/R1 40-47/79-86 (Fig. 6c and 6d). In contrast, the other rabbit in the group (rabbit 134) responded to AF/R1 40-47/79-86, but not to AF/R1 108-123 (Fig. 6d and 6c).

Response of MLN cells to the antigens of AF/R1. Studies have shown that cells undergoing blastogenesis

in the MLN also tend to home into mucosal areas, but experiments requiring in vitro lymphocyte proliferation of rabbit MLN cells are difficult to conduct and to interpret due to non-specific high background cpm in the media controls. Our studies have shown that this problem can be avoided by conducting the proliferative studies in 24-well plates, and then moving aliquots of cells into 96-well plates for pulsing with [^{3}H]thymidine as described in materials and methods. This method of culture was employed for the remainder of the studies. The MLN cells of all rabbits demonstrated a significant proliferation in vitro in response to AF/R1 pili regardless of whether they had been immunized with microencapsulated or non-encapsulated AF/R1 (Fig. 15). However, only the rabbits which had received microencapsulated AF/R1 were able to respond to the AF/R1 synthetic peptide 40-55 (Fig. 11). The MLN cells of rabbit 134 also responded to AF/R1 79-94 ($p<0.0001$), AF/R1 108-123 ($p<0.0001$), and AF/R1 40-47/79-86 ($p=0.0004$); however, none of the other rabbits demonstrated a MLN response to those three peptides (data not shown).

Response of spleen cells to the antigens of AF/R1. Proliferative responses of spleen cells to AF/R1 were very weak in all animals tested (data not shown). However, in results which paralleled the

responses in MLN cells, there was a significant response to AF/R1 40-55 in rabbits which had been primed with microencapsulated AF/R1 (Fig. 12). There was no response to the other AF/R1 synthetic peptides by spleen cells in either group of animals. The weak response of spleen cells to AF/R1 provides further evidence that these animals were naive to AF/R1 before the study began, and indicates that the observed responses were not due to non-specific stimulative factors such as lipopolysaccharide.

XI. SUMMARY

We have shown that there is an enhanced *in vitro* proliferative response to both protein and its peptide antigens by rabbit Peyer's patch cells following intraduodenal inoculation of antigen which had been homogeneously dispersed into the polymeric matrix of biodegradable, biocompatible microspheres. The immunopotentiating effect of encapsulating purified AF/R1 pili as a mucosal delivery system may be explained by one or more of the following mechanisms: (a) Microencapsulation may help to protect the antigen from degradation by digestive enzymes in the intestinal lumen. (b) Microencapsulation has been found to effectively enhance the delivery of a high concentration of antigen specifically into the Peyer's patch. (c) Once inside the Peyer's patch,

microencapsulation appears to facilitate the rapid phagocytosis of the antigen by macrophages, and the microspheres which are 5-10 micrometers become localized within the Peyer's patch. (d) Microencapsulation of the antigen may improve the efficiency of antigen presenttion by decreasing the amount of enzymatic degradation that takes place inside the macrophage before the epitopes are protected by combining with Class II major histocompatibility complex (MHC) molecules. (e) The slow, controlled-release of antigen may produce a depot effect that mimics the retention of antigen by the follicular dendritic cell. (f) If the antigen of interest is soluble, microencapsulation changes the antigen into a particulate form which appears to assist in producing an IgA B cell response by shifting the cellular immune response towards the T_H and thereby not encouraging a response by the T_s. There is evidence that the GALT may be able to discriminate between microbial and non-microbial (food) antigens in part by the form of the antigen when it is first encountered, and thus bacterial antigens do not necessarily have special antigenic characteristics that make them different from food antigens, but they are antigenic because of the bacterial context in which they are presented. The particulate nature of microspheres may

serve to mimic that context. It may be important to note that we also observed a significant response to AF/R1 in animals inoculated with non-encapsulated pili; thus, some of this antigen which was still in its native form was able to enter the Peyer's patch. This may be explained by the fact that AF/R1 is known to mediate the attachment of RDEC-1 to the Peyer's patch M-cell. If the antigen employed in this type of study was not able to attach to micrometer M-cells, one would expect to see an even greater difference in the responses of animals which had received microencapsulated versus non-encapsulated antigen.

The microspheres used in these experiments included a size range from 1 to 12 micrometers. The 1 to 5 micrometer particles have been shown to disseminate to the MLN and spleen within migrating macrophages; thus, the observed proliferative responses by cells from the MLN and spleen may reflect priming of MLN or splenic lymphocytes by antigen-presenting/accessory cells which have phagocytosed 1 to 5 micrometer antigen-laden microspheres in the Peyer's patch and then disseminated onto the MLN. Alternatively, these responses may be a result of the normal migration of antigen stimulated lymphocytes that occurs from the Peyer's patch to the MLN and on into the general circulation before homing

to mucosal sites. Proliferative responses by MLN cells are of interest because it has been shown that cells undergoing blastogenesis in the MLN tend to migrate onto mucosal areas. However, studies involving in vitro lymphocyte proliferation of rabbit MLN cells can be very difficult to conduct and to interpret due to non-specific high background cpm in the media controls. By simultaneously conducting experiments using different protocols, we have found that this problem can be prevented by avoiding the use of fetal calf serum in the culture and by initially plating the cells in 24-well plates. Using this method, the blasting lymphocytes are easily transferred to a 96-well plate where they receive the [^{3}H]thymidine, while fibroblasts and other adherent cells remain behind and thus do not inflate the background cpm.

The proliferative response to the peptide antigens was of particular interest in these studies. The rabbits that received non-encapsulated AF/R1 failed to respond to any of the peptides tested either at the level of the Peyer's patch, the MLN, or the spleen. In contrast, Peyer's patch cells from the animals that received microencapsulated AF/R1 responded to all the peptides tested with two exceptions: Rabbit 134 did not respond to AF/R1 108-123, and rabbit 135 did not respond to AF/R1 40-47/79-86. The reason for these

non-responses is not clear, but it probably is not due to MHC restrictions as evidenced by the fact that rabbit 134 was able to respond to AF/R1 108-123 at the level of the MLN. The non-responses may be due to varing kinetics of sensitized T cell migration in different rabbits, or they may reflect differences in the efficiency of antigen presentation by cells from different lymphoid tissues of these animals. Of all the synthetic peptides tested, only AF/R1 40-55, (the one selected as a probable B cell epitope), was recognized by serum from an AF/R1 hyperimmune rabbit. In addition, this peptide was the only one that was uniformly recognized by Peyer's patch, MLN, and spleen cells from both rabbit. In addition, this peptide was the only one that was uniformly recognized by Peyer's patch, MLN, and spleen cells from both rabbits that were immunized with microencapsulated AF/R1. The recognition by anti-AF/R1 serum antibodies indicates that the amino acid sequence of this peptide includes an immunodominant B cell epitope. Thus AF/R1 40-55 may readily bind to antigen-specific B cells thereby leading to an efficient B cell presentation of this antigen to sensitized T cells. Even though AF/R1 40-55 was not selected as a probable T cell epitope by either the Rothbard or Berzofsky methods, the current study clearly indicates that this peptide can also stimulate

a proliferative immune response. Although further studies are required to definitively show that the proliferating cells are indeed T cells, the responses observed in this study are most likely due to the blast transformation of cells from the lineage. Therefore, AF/R1 40-55 appears to contain a T cell epitope in addition to the immunodominant B cell epitope, and this area of the AF/R1 protein may thereby play an important role in the overall immune response and subsequent protection against RDEC-1.

The proliferative responses of spleen cells was low in all animals tested; however, we feel tht this may be simply a matter of the kinetics of cellular migration. The rabbits in this study were sacrificed only two weeks after their first exposure to antigen. This relatively short time period may not have provided sufficient time for cells that were produced by Peyer's patch and MLN blasts to have migrated as far as the spleen in sufficient numbers.

An ideal mucosal vaccine preparation would not only assist in the uptake and presentation of the immunogen of interst, but it would also be effective without requiring carrier molecules or adjuvants which may complicate vaccine production or delay regulatory approval. The incorporation of antigen into microspheres appears to provide an ideal mucosal

delivery system for oral vaccine immunogens because the observed immunopotentiating effect is achieved without the need for carriers of adjuvants. This ability may prove to be of great value, particularly to enhance the delivery of oral synthetic peptide vaccines to the GALT.

TABLE 1. Linear B-Cell Epitopes of CFA/I in Monkeys

	Sequence Position	Individuals Responding	Consensus Site
1.	11-21	3	VDPVIDLLQ
2.	93-101	2	AKEFEAAA
3.	124-136	2	GPAPT
4.	66-74	2	PQLTDVLN
5.	22-29	2	GNALPSAV
6.	32-40	1	KTF*
7.	38-45	1	
8.	3-11	1	

*Overlap between epitope 6 and 7

TABLE 2

Prediction of T cell epitopes within the CFA/I molecule[a]

	Predicted Amphipathic Segments		Rothbard Criteria
	7 aa blocks	11 aa blocks	
	22-25	8-11	
16			
	34-38	32-44	
30			
	40-46	51-71	
38			
	50-53	86-92	
44			
	56-62	102-108	
57			
	64-71	130-131	
61			
	104-108	135-137	
70			
	131-137		
116			
			124
			127
			137

[a]The sequence numbers of the first amino acid of the predicted T cell epitopes are shown. Software designed to predict T cell epitopes based on the Berzofsky method was published as the AMPHI program. It predicts amphipathic amino acid segments by evaluating 7 or 11 residues as a block and assigning a score to the middle residue of that block. Software designed to predict T cell epitopes based on the Rothbard method was written by Stephen Van Albert (The Walter Reed Army Institute of Research, Washington, D.C.).

TABLE 3

Amino acid sequence of immunodominant T cell epitopes[a]

Residue Numbers	Amino Acids
8-17	Thr Ala Ser Val Asp Pro Val Ile Asp Leu
40-49	Phe Glu Ser Tyr Arg Val Met Thr Gln Val
72-81	Leu Asn Ser Thr Val Gln Met Pro Ile Ser
134-143	Asn Tyr Ser Gly Val Val Ser Leu Val Met

[a]Of the 19 decepeptides that supported a significant proliferative response and contained a serine at either position 2, 3, or 4, nine has a serine specifically at position 3. Some of the most robust

responses were to the peptides that contained a serine residue at the third position. The amino acid sequence of four such decapeptides which are believed to be immunodominant T cell epitopes is shown.

PHASE III

The development of a safe and effective vaccine against enterotoxigenic E. coli (ETEC) would be useful for travelers and for young children in endemic areas. A phase I study of an enteral ETEC vaccine candidate consisting of colonization factor antigen II (CFA/II) encapsulated in biodegradable polymer microspheres (BPM) was conducted in healthy volunteers.

Ten adult volunteers swallowed intestinal tubes on days 0, 7, 14, and 28; after collection of jujunal fluid samples, 1 mg of CFA/II in BPM was administered via the tube. Volunteers kept a diary of symptoms after each dose. Secretory IgA in jejunal fluids, serum responses, and antibody secreting cells (ASC) were measured before and after vaccination.

The vaccine was well tolerated. Five of 10 volunteers had developed IgA anti-CFA/II ASC by 7 days after the last dose of vaccine, these same 5 vaccinees had IgA anti-C63 ASC, and 3 of 5 vaccinees had IgA anti-CS1 ASC. Five of 10 vaccinees developed rises in jejunal fluid sigA anti-CFA/II with peak CMT of 1:42. Serum responses were meager. Ten vaccinees and 10

unvaccinated control volunteers underwent challenge with 10^9 cfu ETEC E24377A (0139;H2B LT+ST+CS1+CS3+). Ten of 10 controls and 7 of 10 vaccinees developed diarrhea (p=.11, 30% vaccine efficacy). One of the 3 protected vaccinees had the highest number of ASC and highest sIgA titer before challenge, suggesting that these responses were protective and that this vaccine development strategy has merit. Future studies with higher dosages and a different dosing schedule are planned.

Enterotoxigenic Escherichia coli (ETEC) is responsible for diarrhea in infants in developing countries and for a large proportion of diarrhea among travelers to developing countries. Development of a vaccine against ETEC is therefore an important public health priority. Studies in animals and challenged volunteers suggest that orally administered fimbriae, which function as colonization factors, should induce protective immunity.

An ETEC vaccine candidate was developed which consists of purified colonization factor antigen II (CFA/II) derived from ETEC strain M424 (06:H16:K15) encapsulated in biodegradable polymer microspheres (BPM). CFA/II from this strain consists of two surface structures, a fibrillar designated coli surface antigen 1 (CS1) and a fibrillar structure designated coli

surface antigen 3 (CS3). The purpose of encapsulating the antigen into microspheres is to protect it during passage through the stomach and to enhance its uptake by gut-associated lymphoid tissues (GALT), such as Peyer's patches. The microspheres consist of a 50:50 copolymer of lactic and glycolic acids (DL-lactide-co-gylcolide). In animals, antigens delivered in these microspheres are taken up and processed by the GALT and stimulate vigorous local immune responses.

In this report we describe the safety, Immunogenicity, and efficacy against experimental challenge of the CFA/II-BPM vaccine in healthy volunteers. This is the first use in man of this delivery system for an oral antigen.

This phase III describes the result of E. coli CVD 15000, a clinical study of the safety, immunogenicity, and efficacy against experimental challenge of a new vaccine against enterotoxigenic E. coli (ETEC). This vaccine consists of colonization factor antigen II (CFA/II) purified from ETEC strain M424 (06:H16:K15) encapsulated in biodegradable polymer microspheres (CFA/II-BPM).

MATERIALS AND METHODS

CFA/II-BPM vaccine was prepared at the University of Maryland School of Pharmacy. Each dose of vaccine consisted of 1 mg of CFA/II (90% CS3, 10% CS1)

incorporated into 100 mg of BPM 1. 10 microns in diameter; the freeze-dried microspheres were dispersed in saline containing 0.5% polysorbate$_{60}$. Ten healthy adult outpatient volunteers were recruited for vaccination with four doses of CFA/II-BPM vaccine. Each volunteer swallowed an intestinal tube on days 0, 7, 14, and 28; after collection of jejunal fluid, CFA/II-BPM was administered via the tube. The vaccine vials were sonicated immediately before vaccination to achieve an even suspension of the turbid vaccine.

Volunteers kept a diary of symptoms for five days after each dose of vaccine. Jejunal fluids were collected via intestinal tube on days 0, 7, 14, 28, and 35 after vaccination for measurement of secretory IgA. Whole blood for antibody secreting cells (ASC) was collected on days 0, 7, 14, 21, and 35. Serum was collected for antibody against CFA/II on days 0, 7, and 28. ASC responses were measured by ELISPOT assays using a variety of antigens: CFA/II vaccine antigen derived from ETEC strain M424 (06:H16:K15 CS1+CS3+), purified CS1 derived from ETEC strain 60R75 (O:H CS1+), and purified CS3 derived from ETEC strain E9034 (08:H9 CS3+). Four or more spots was considered a significant number. Serum antibody measurements to CFA/II, purified CS1, and purified CS3 were performed by ELISA. A four-fold rise in titer was considered significant.

Jejunal fluids were adjusted to a concentration if IgA of 20 mg% and then lyophilized before assaying for specific anti-CFA/II activity.

Fifty-seven days after the first dose of CFA/II-BPM vaccine, 10 vaccinees and 10 unimmunized control volunteers were admitted to the Research isolation Ward in the University of Maryland Hospital. AFter screening for excellent health, volunteers ingested 3 x 10^9 cfu of ETEC strain E24377A (O139:H28 LT+ST+CS1+CS3+) with sodium bicarbonate. Blood samples were collected for serologic responses to CFA/II, O139(LPS) antigen, and heat labile enterotoxin (LT) before and on days 14 and 28 after challenge. Jejunal fluids for measurement of sigA against CFA/II were collected before and on day 7 after challenge.

Part I: Outpatient Vaccination Study

Ten healthy adult outpatients volunteers were recruited for vaccination with CFA/II-BPM vaccine. Each volunteer swallowed an intestinal tube on September 2, 9, 16, and 30 (days 0, 7, 14, and 28); after collection of jejunal fluid, 1 mg of CFA/II in BPM was administered via the tube. The vaccine was prepared immediately before vaccination as directed by Dr. Reid; specifically, the vials were sonicated to achieve an even suspension of the turbid vaccine. For two volunteers, one or more doses of vaccine had to be

administered intragastrically (noted in data tables) because the tube failed to move out of the stomach after over 56 hours of intubation.

Safety. Volunteers kept a diary of symptoms for five days after each dose of vaccine. The vaccine was well tolerated. One volunteer reported mild cramps for 15 minutes on day 1 after the second dose. A second volunteer reported cramps lasting for about one hour before passing loose stools on days 3 and 4 after teh second dose; the volunteer attributed this to having eaten crabs.

Immunogenicity. Jejunal fluids were collected via intestinal tube on days 0, 8, 14, 28, and 35 after vaccination for measurement of secretory IgA. Whole blood for antibody secreting cells (ASC) was collected on days 0, 7, 14, 21, and 35. Serum was collected for antibody determinations on days 0, 7, and 28. Whole blood for measuring T cell responses by lymphocyte transformation were drawn on days 0 and 35 after vaccination.

ASC. Detection of CFA/II-specific antibody secreting cells in peripheral blood reflects priming of the intestnal mucsal immune system; these cells have been stimulated by oral antigen, entered the circulation, and are returning to the mucosa to provide

local immunicyt. The role of these cells in protection against ETEC diarrhea is unknown.

We measured ASC responses by ELISPOT assasys using a variety of antigens: CFA/II vaccine antigen derived from ETEC strain M424 (06:H16:K15 CS1+CS3+), purified CD1 derived from ETEC strain 60R75 (0:H CS1+), purified CS3 derived from ETEC strain E9034 (08:H9 CS3+), CS3 peptide 795, CS3 peptide 792, and as controls, CFA/I, CFA/I peptide 791, and CFA/I peptide 900. The results of these assays are shown in Tables 1 through 5. Four or more spots is considered a significant number.

At day 7 after the first dose of vaccine, four of the 10 volunteers developed IgA ASC against CFA/II (Table 1). After the second and thrid doses of vaccine no additional responders were detected. However, after the fourth dose, an additional volunteer developed a significant response so that the overall response after four doses of CFA/II-BPM was five (50%) of 10 vaccinees.

Three of the volunteers who responded with IgA ASC against CFA/II also had IgA ASC against purified CS1 (Table 8). The same five volunteers who responded to CFA/II also had IgA ASC against purified CS3 (Table 9). The suggests that the responses to CFA/II were specific and not directed against contaminating

elements such as LPS, since the serotypes of the strains from which the antigens were prepared were different. IgA ASC responses to two peptides derived from CS3 were meager or absent (Tables 10 and 11). There were no ASC responses to to CFA/I or to two peptides derived from CFA/I. This is further evidence that the responses to CFA/II were not directed against contaminating elements in the antigen preparations.

Jejunal fluid sigA. After the first dose of CFA/II-BPM vaccine, only one volunteer developed a rise in sigA to CFA/II and this volunteer (15001-9) had evidence of previous priming since his pre-vaccination sigA anti-CFA/II titer was 1:16 (Table 12). One week after the fourth dose (day 35), however, five of the 10 vaccinees had developed rises in sigA anti-CFA/II. Among these five converters, the peak geometric mean titer was 1:42.

Serology. Serum antibody meansurements to CFA/II, purified CS1, and purified CS3 were also performed by ELISA. A four-fold rise in titer was considered significant and indicated by a + in the tables. There was a high prevalence of serum antibody to CFA/II before vaccination (Table 13); only two of 10 volunteers developed rises in serum IgA anti-CFA/II and a third volunteer developed a rise in serum IgG anti-CFA/II. Only one volunteer developed serum antibody to

CS1 (Table 14). However, six of the 10 vaccinees developed seroconversions to anti-CS3 with antibody of at least one isotpy (Table 15).

Lymphocyte proliferation studies. Lymphocytes were separated from whole blood on ficoll-hypaque gradients and stored forzen for future proliferative assays by Dr. Reid at WRAIR.

Part II: Experimental ETEC Challenge Study

All 10 vaccinees and 10 control volunteers agreed to participate in an ETEC challenge. One October 29, 1992, 57 days after the first dose of CFA/II-BPM vaccine, 20 volunteers ingested 3 x 10^9 cfu of ETEC strain E24377A (0139:H28 LT+ST+CS1+CS3+). The clinical and bacteriologic responses to challenge are shown in Table 10.

Ten of 10 control volunteers and seven of 10 vaccines developed diarrhea (p=0.11, Fisher's exact test, 1-tailed; 30% vaccine efficacy). the mean volume of diarrheal stools was 1464 ml for controls and 2819 ml for vaccines (p=0.2, Student's t test); the mean number of diarrheal stools was 8.6 for controls and 14.7 for vaccinees (p=0.2, Student's t test). The mean incubation periods in the two groups were not significantly different. The duration of stool shedding and the peak stool excretion of challenge organisms were not significantly different. The three

protected vaccinees had a somewhat lower peak excretion of challenge organisms than the seven unprotected vaccinees, but this difference was not statistically significant.

Before challenge (day 57 after the first dose of vaccine), the three protected vaccinees, five vaccinees who became ill, and four control volunteers who became ill had circulating ASC producing antibodies of some isotype against CFA/II, CS1, or CS3 (Table 17). The vaccinee (volunteer 15001-9) with the highest number of IgA anti-CFA/II ASC (240 spots) before challenge and the highest number of IgA anti-CS3 ASC (16 spots) before challenge was one of the three protected vaccinees. The other protected vaccinees (volunteers 15001-6 and 15001-11) had no detectable anti-CFA/II IgA ASC before challenge but did have anti-CS1 ASC or anti-CS3 IgA ASC. Conversely, unvaccinated control volunteers with pre-existing IgA anti-CFA/II ASC were not protected (e.g., volunteers 15002-8, 15002-11, and 15002-13).

The level of ASC response inducted by infection provides a target for future vaccine-induced immunity. After wild-type challenge of vaccinees and controls, IgA ASC responses to CFA/II and CS3 were vigorous (range 12-408 spots for CFA/II and 14-712 spots for CS3) (Table 17). After challenge one vaccinee and one

control volunteer mounted ASC responses to CS3 peptide 792 (Table 18). Four vaccinees (15001-1, 15001-6, 15001-7, and 15001-11) and one control volunteer (15002-11) developed a small number of ASC to CS3 peptide 795 (Table 18).

There was no correlation between pre-existing anti-LPS ASC and protection (Table 19). None of the three protected vaccinees had such antibodies before challenge. Two volunteers with pre-existing anti-LPS ASC nevertheless became ill (volunteers 15001-1 and 15001-8). Similarly, there was not correlation between protection against illness and pre-existing anti-LT ASC (Table 19).

The serologic responses and jejunal fluid antibody responses to challenge are pending at the time of this writing. These results will be summarized in an addendum to this report.

RESULTS

Clinical and immunologic responses to vaccination. The vaccine was well tolerated. For two volunteers, four doses of vaccine had to be administered intragastrically in two volunteers because the tube failed to move out of the stomach after over 56 hours of intubation.

Detection of CFA/II-specific antibody sacreting cells in perlpheral blood reflects priming of the

intestinal mucosal immune system; these cells have been stimulated by oral antigen, entered the circulation, and are refurring to the mucosa to provide local immunity. At day 7 after the first dose of vaccine, four of the 10 volunteers developed IgA ASC against CFA/II. Ater the second and third doses of vaccine no additional responders were detected. However, after the fourth dose, an additional volunteer developed a significant response so that the overall response after four doses of CFA/II-BPM was five (50%) of 10 vaccinees by day 35 (Table 20). Three of the volunteers who responded with IgA ASC against CFA/II also had IgA ASC against purified CS1 (Table 20). The same five volunteers who responded to CFA/II also had IgA ASC against purified CS3 (Table 20). This suggests that the responses to CFA/II were specific and not directed against contaminating elements such as LPS, since the serotypes of the strains from which the antigens were prepared were different.

After the first dose of CFA/II-BPM vaccine, only one volunteer developed a rise in jejunal fluid sigA to CFA/II, and this volunteer had evidence of previous priming since his pre-vaccination sigA anti-CFA/II titer was 1:16. One week after the fourth dose (day 35), however, five of the 10 vaccinees had developed

rises in sigA anti-CFA/II (Table 20). Among these five converters, the peak geometric mean titer was 1:42.

There was a high prevalence of serum antibody to CFA/II before vaccination; only two of 10 volunteers developed rises in serum IgA anti-CFA/II and a third volunteer developed a rise in serum IgG anti-CFA/II. Only one volunteer developed serum antibody to CS1. However, six of the 10 vaccinees developed seroconversions to anti-CS3 with antibody of at least one isotype.

Clinical and bacteriologic responses to experimental ETEC challenge. Fifty-seven days after the first dose of CFA/II-BPM vaccine, 10 vaccinees and 10 control volunteers ingested 3 x 10^9 cfu of ETEC strain E24377A (0139:H28 $LT^+ST^+CS1^+CS3^+$). The immunologic status at the time of challenge and the clinical and bacteriologic responses to challenge are shown in Table 22.

Ten of 10 control volunteers and seven of 10 vaccinees developed diarrhea (p=0.11, Fisher's exact test, 1-tailed; 30% vaccine efficacy). The mean volume of diarrheal stools was 1464 ml for controls and 2819 ml for vaccines (p=0.2, Student's t test); the mean number of diarrheal stools was 8.6 for controls and 14.7 for vaccinees (p=0.2, Student's t test). The mean incubation periods in the two groups were not

significantly different. The duration of stool shedding and the peak stool excretion of challenge organisms were not significantly different.

On the day of challenge, 8 of 10 vaccinees and 4 of the 10 control volunteers had circulating IgA ASC producing antibodies against CFA/II, CS1, and/or CD3. The apparent development of additional ASC responders on day 57 after the first dose of vaccine (making the total number of vaccine responders 8 of 10) was unexpected. The high prevalence of anti-colonization factor ASC in control volunteers before challenge was also unexpected and not observed in previous groups of North American volunteers. The vaccinees with the highest number of IgA anti-CFA/II ASC (240 spots), the highest number of IgA anti-CS3 ASC (16 spots), and the highest sigA anti-CFA/II tirer (1:256) before challenge was one of the three protected vaccinees. Conversely, the 4 unvaccinated control volunteers with pre-existing IgA anti-CFA/II ASC (range 8-32 spots) were not protected; none of these had pre-existing sigA measured in jejunal fluid before challenge.

There was no correlation between pre-existing anti-LPS ASC and protection. Similarly, there was no correlation between protection against illness and pre-existing anti-LT ASC.

Immune responses after wild-type challenge, which are likely to be protective against subsequent challenge, are a target for vaccine-induced immunity. The immune responses in volunteers after 4 doses of CFA/II-BPM vaccine (Table 20) can be compared to those of unimmunized control volunteers after challenge (Table 21). Responses after this vaccine regimen occurred at a lower rate and were of lower magnitude than those achieved after a vigorous wild-type challenge.

DISCUSSION

CFA/II-BPM vaccine was well tolerated in adult volunteers. When immune responses were measured by the presence of IgA ASC or jejunal fluid sigA, both measured 7 days after the fourth dose, half the volunteers responded to four doses of 1 mg CFA/II-BPM per dose. The vaccine conferred 30% protective efficacy against a rigorous experimental challenge that produced an attach rte of 100% in control volunteers.

The three protected vaccinees did not differ significantly from the seven unprotected vaccinees, at least in the immune parameters measured in this study. However, the volunteer who had the highest number of ASC against CFA/II and CS3 and the highest sigA titers among the vaccinees was one of the 3 vaccinees who did

not become ill. This suggests that these immune responses contributed to protection.

Some volunteers had a significant number of IgA, IgG, or IgM ASC to CFA/II and/or CS3 on day 57 after the first dose of vaccine (the day of challenge) that were not present on day 35 after vaccination. This suggests that the biodegrabable polymer microspheres may have persisted in the submucosa and continued to stimulate responses beyond the 7 to 10 days when ASC responses are ordinarily expected. However, some control volunteers also had ASC responses to CFA/II before challenge. No technical difficulty with the ASC assay could be identified and control blank wells did not react. Confirmation of the presistence of CFA/II-BPM vaccine with continued induction of immune responses will await future studies.

The modest efficacy of CFA/II-BPM vaccine may be related to the very small doseage (1 mg of CFa/II x 4 doses) given. The responses after ETEC challenge summarized in Table 21. However, are within reach, perhaps by increasing the dose or changing the schedule of vaccination. Future studies should also include evaluation of the oral route of administration because of the impracticality of delivering vaccine via intestinal tube.

DEMONSTRATIVE EVIDENCE OF PROTECTIVE IMMUNITY

RDEC-1 is an eteroadherent diarrhea producing E. coli in rabbit. Its attachment to the mucosa is by the adhesin (AF/R1 pili). The adhesin is an excellent vaccine candidate. It may intitiate a mucosal response but is susceptiple to digestion in the gut. The incorporation of AF/R1 into biocompabible, nondigestible microspheres enhanced mucosal cellular immune respones to RDEC-1. We have demonstrated that immunization with AF/R1 Pili in microspheres protect rabbits against infection with RDEC-1.

Six rabbits received intra-duodenal immunizaiton of AF/R1 microspheres (0.62% coreloading by weight) at 200 ug AF/R1 on day 0 then boosted with 100 ug AF/R1 in microspheres on days 7, 14, and 21 followed by RDEC-1 challenge with 10^8 organisms one week latter than observed for 1 week and then sacrificed, unimmunized rabbits were challenged with 10^8 RDEC-1 only and observed 1 week than sacrified. Also, 2 rabbits were immunized only then were sacrificed 10 days latter. Only one of these animals had bile IgA antibodies to AF/R1. but both had specific sensitized T cells which released IL-4 upon challenge in the spleen, Peyer's patch and illeal lamina propria. All nine immunized animals developed diarrhea and weight loss which was significant at the $p < .001$ level compared to the immunized animals which displayed no diarrhea and no

weight loss. The immunized animals colonized the intestinal tract with RDEC-1 the same as the unimmunized animals. However, there was a striking difference regarding the adherence of RDEC-1 to the mucosa. No adherence was seen in cecum in the immunized animals compared to 4/7 in the unimmunized side animals. This difference was significant to the $p < .01$ level. The RDEC-1 exposure although not producing disease in the immunized animals did effect a booster immunization as relected in the increase in anti-AF/R1 antibody containing cells in the muscosa similiar to the immunized rabbits. This study clearly demonstrated complete protection against RDEC-1 infection and strongly indicates similiar results should be expected with entertoxigenicity E. coli using the Colony Forming Antigens (CFA's) in microsphere vaccines.

SUMMARY STATEMENT OF PROTECTIVE IMMUNITY SHOWINGS

RDEC-1 infection of rabbits causes an enteroadherent E. coli diarrheal disease, and provides a model for the study of adherence-factor immunity. Pilus adhesions are vaccine candidates, but purified pili are subject to intestinal degradation. Previously we showed potentiation of the mucosal cellular immune response to the AF/R1 pilus of RDEC-1 by incorporation into biodegradable polylactide-coglycolide microspheres

(AF/R1-MS). We now present efficacy testing of this vaccine. Six rabbits were primed with 200 ug and boosted with 100 ug of AF/R1-MS weekly x3, then challenged at week 5 with 10^8 CFU of RDEC-1 expressing AF/R1. Nine unvaccinated rabbits were also challenged. Two rabbits vaccinated with AF/R1-MS were sacrificed at week 5, without challenge, for measurement of anti-AF/R1 antibodies in bile (by ELISA) and anti-AF/R1 containing cells (ACC) in the intestinal lamina propria (by immunohistochemistry). Attachment of RDEC-1 to intestinal epithelial cells was estimated (0.4+) by immunoperoxidase staining of histologic sections. Colonizaiton of intestinal fluid was measured by culture of intestinal flushes. Results: Rabbits given AF/R1-MS remained well and 4/6 gained weight after challenge, whereas 9/9 unvaccinated rabbits lost weight after challenge (mean weight change +10 vs -270 gms $p<.001$), (see Figure 27). The mean score of RDEC-1 attachment to the cecal epithelium was 0 in vaccinated, and 2+ in unvaccinated animals (see Figure 28). RDEC-1 colonizaiton (log CFU/gm) in cecal fluids was similar in both groups (mean 6.3 vs 7.3; $p=.09$) (see Figure 26).. ACC were not seen in the lamina propria of vaccinated but unchallenged animals, but anti-pilus IgA antibody levels in bile were increased 1 S.D. over negative controls in 1 animal. Conclusions:

Vaccination with AF/R1-MS was safe and protected rabbits against RDEC-1 disease. Protection was associated with interference with RDEC-1 adherence to the mucosal surface, but lumenal colonization was not prevented.

More recently, applicants have focused on areas of this invention related to an immunostimulating composition comprising encapsulating microspheres, which may contain a pharmaceutically-acceptable adjuvant, wherein said microspheres are comprised of (a) a biodegradable-biocompatible poly (DL-lactide-co-glycolide) as the bulk matrix, wherein the relative ratio between the amount of lactide and glycolide components are within the range of 40:60 to 0:100 and (b) an immunogenic substance comprising Colony Factor Antigen (DFA/II, hepatitis B surface antigen (HBsAg), or a physiologically similar antigen that serves to elicit the production of antibodies in mammalian subjects.

These areas of invention are referred to herein after as Phase III and Phase IV, respectively, and are summarized as follows:

1. An immunostimulating composition comprising encapsulating- microspheres, which may contain a pharmaceutically-acceptable adjuvant, wherein said microspheres having a diameter between 1 nanogram (ng)

to 10 microns (um) are comprised of (a) a biodegradable-biocompatible poly (DL-lactide-co-glycolide) as the bulk matrix, wherein the relative ratio between the amount of lactide and glycolide components are within the range of 40:60 to 0:100 and (b) an immunogenic substance comprising Colony Factor Antigen (CFA/II), hepatitis B surface antigen (HBsAg), or a physiologically similar antigen that serves to elicit the production of antibodies in animal subjects.

2. An immunostimulating composition according to Claim 1 wherein the amount of said immunogenic substance is within the range of 0.1 to 1.5% based on the volume of said bulk matrix.

3. An immunostimulating composition according to Claim 2 wherein the relative ratio between the lactide and glycolide component is within the range of 48:52 to 58:42.

4. An immunostimulating composition according to Claim 2 wherein the size of more than 50% of said microspheres is between 5 to 10 um in diameter by volume.

5. An immunostimulatiang composition according to Claim 1 wherein the immunogenic substance is the synthetic peptide representing the peptide fragment beginning with the amino acid residue 63 through 78 of Pilus Protein CS3, said residue having the amino acid sequence, 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-His-Glu-Thr-Asn-Asn-Ser-Ala).

6. A vaccine comprising an immunostimulating composition of Claim 4 and a sterile, pharmaceutically-acceptable carrier therefor.

7. A vaccine comprising an immunostimulating composition of Claim 6 wherein said immunogenic substance is Colony Factor Antigen (CFA/II).

8. A vaccine comprising an immunostimulating composition of Claim 6 wherein said immunogenic substance is hepatitis B surface antigen (HBsAg).

9. A method for the vaccination against bacterial infection comprising administering to a human, an antibactericidally effective amount of a composition of Claim 6.

10. A method according to Claim 8 wherein the bacterial infection is caused by a bacteria selected from the group consisting essentially of Salmonella typhi, Shigella Sonnei, Shigella Flexneri, Shigella dysenteriae, Shigella boydii, Escheria coli, Vibrio cholera, yersinia, staphylococus, clostridium, and campylobacter.

11. A method for the vaccination against viral infection comprising administering to a human an antivirally effective amount of a composition of Claim 8.

12. A diagnostic assay for bacterial infections comprising a composition of Claim 4.

13. A method of preparing an immunotherapeutic agent against infections caused by a bacteria comprising the step of immunizing a plasma donor with a vaccine according to Claim 7 such that a hyperimmune globulin is produced which contains antibodies directed against the bacteria.

14. A method preparing an immunotherapeutic agent against infections caused by a virus comprising the step of immunizing a plasma donor with a vaccine

according to Claim 8 such that hyperimmune globulin is produced which contains antibodies directed against the hepatitis B virus.

15. An immunotherapy method comprising the step of administering to a subject an immunostimulatory amount of hyperimmune globulin prepared according to Claim 13.

16. An immunotherapy method comprising the step of administering to a subject an immunostimulatory amount of hyperimmune globulin prepared according to Claim 14.

17. A method for the protection against infection of a subject by enteropathogenic organisms or hepatitis B virus comprising administering to said subject an immunogenic amount of an immunostimulating composition of Claim 3.

18. A method according to Claim 17 wherein the immunostimulating composition is administered orally.

19. A method according to Claim 17 wherein the immunostimulating composition is administered parenterally.

20. A method according to Claim 17, wherein the immunostimulating composition is administered in four separate doses on day 0, day 7, day 14, and day 28.

21. A method according to Claim 17 wherein the immunogenic substance is the synthetic peptide representing the peptide fragment beginning with the amino acid residue 63 through 78 of Pilus Protein CS3 said residue having the amino acid sequence 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-His-Glu-Thr-Asn-Asn-Ser-Ala).

PHASE III

In sum, the Colony Factor Antigen (CFA/II) from enterotoxigenic E coli (ETEC) prepared under GMP was successfully incorporated into biodegradable polymer microspheres (CFA/II BPM) under GMP and found to be safe and immunogenic when administered intra-duodenally to rabbits. CFA/II was incorporated into poly (D,L-lactide-co-glycolide) (PLGA) microspheres which were administered by direct endoscopy into the duodenum. Following vaccination, Peyer's patchcells responded by lymphocyte proliferation to in vitro challenge with CFA/II indicating the CFA/II BPM to be immunogenic when administered intra-intestinally. Also, B cells secreting specific anti CFA/II antibodies were found in

spleens following vaccination. No pathological changes were found following total necropsies of 10 rabbits vaccinated with CFA/II BPM. As a potency test, high serum IgG antibody titers to CFA/II were produced following intra- muscular administration of CFA/II BPM to additional rabbits. The CFA/II BPM contained 63% between 5-10 um by volume particle size distribution; 1.17% protein content; 2.15% moisture; <.01% acetonitrile; 1.6% heptane; 22 nonpathogenic bacteria and 3 fungi per 1 mgm protein dose; and passed the general safety test. We conclude that the CFA/II BPM oral vaccine is immunogenic and safe to begin a Phase I clinical safety study following IND approval.

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) causes diarrheal disease with an estimated 650,000,000 cases anually in developing countries resulting in 500,000 deaths predominantly in the pediatric age groups. Currently there is no vaccine against ETEC induced diarrhea. The availability of an effective oral vaccine would be of great value to the people of South America, Africa and and Asia as well as the millions of people who travel to these high risk areas and account for half of the annual cases.

The first step in pathogenesis is adherence to the small intestine epithelial cells by protein fimbrial (pilus) adhesins called colonization factor antigen (CFA). Three major CFAs have been recognized, CFA/I, CFA/II and CFA/IV. (25)

Ten human volunteers who were immunized orally twice weekly for 4 weeks with CFA/II developed a poor antibody response and did not show any significant protection when challenged with pathogenic ETEC (26). This disappointing response was attributed to adverse effects of gastric acid, even at neutral pH, of fimbrial proteins (27). When the vaccine was administered by inoculation directly into the duodenum, 4 of 5 immunized volunteers developed a significant rise in secretory IgA with CFA/II antibody (26).

D and L-lactic acid and glycolic acid, as homo- and copolymers, are biodegradable and permit slow and continued release of antigen with a resultant adjuvant activity. These polymers have been shown to be safe in a variety of applications in human beings and in animals (28-32). Delivery of antigens via microspheres composed of biodegradable, biocompatible lactide/ glycolide polymers (29-32) may enhance the mucosal response be protecting the antigen from digestion and

targeting them to lymphoid cells in Peyer's patches (29-32). McQueen et al. (33) have shown that E coli AF/R1 pili in PLGA microspheres, introduced intra-duodenally in rabbits, protected them against diarrhea and weight loss when challenged with the parent strain rabbit diarrheagenic strain of E coli (RDEC-1). Only one vaccinated rabbit of six lost weight and only one had soft pelleted stool. In contrast, all control unvaccinated animals became ill, lost weight, and shed soft pellets or unformed mucoid stool. Significant lymphocyte proliferation to AF/R1 from Peyer's patches and ordinary IgA anti AF/R1 antibody levels were seen.

In order to improve the CFA/II vaccine it was incorporated into PLGA microspheres under GMP in order to protect it from digestion and target it to the intestinal lymphoid system. The CFA/II BPM vaccine has undergone pre-clinical evaluation and has been found to be safe and immunogenic.

MATERIALS AND METHODS

Preparation of CFA/II Pilus Vaccine. Under Good Laboratory and Good Manufacturing Practices, E. coli. strain M424C1-06;816 producing CFA/II were cultured in 75-80 CFA agar plates (24 x 24 cm) for 24 hrs then harvested by scraping. The harvest was homogenized at

slow speed for 30 minutes with over head drive unit and cup immersed in an ice bath. The homogenate was centrifuge at 4° C at 16, 500 x g for 30 minutes. The supernatant saved and the pellet rehomogenized and centrifuged with the supernatants pooled. The supernatant pool was centrifuged at 50,000 x g for 45 minutes. The supernatant treated with ammonium sulfate at 20% satuaration, stirred 30 minutes at 4° C than stored at 4° C for 16 hrs then centrifuged at 19,700 x g for 30 minutes. The supernatant saved and treated with ammonium sulfate at 45% saturation, stirred 30 minutes at 4° C, stored at 4° C for 66-72 hrs, then centrifuged at 19,700 x g for 45 minutes. The pellet was resuspended in about 100 mls of PBS containing 0.5% formalin and held at 22° for 18 hrs then dialyzed for 45-50 hrs against PBS at 4° C using a total of 12 liters in 2 liter amounts. The dialysis was terminated when the PBS contained less then 0.03% formalin using Nessler's reagent and fuchsin sulfuose acid reagent. The final product contained 1 mgm protein/ml PBS, was sterile and passed the general safety test.

Preparation of Desalted CFA/II Vaccine. Two ml of the CFA/II vaccine were placed into a Centricon 30 tube and centrifuged at 1700 rpm at 4-6° C (Beckman model GPR centrifuge equipped with GA-24fixed angle rotor) until all the buffer solution passed through the

filter (about 90-120 minutes). Sterile water was added to each tube to disperse the CFA/II retained on the filter. The desalted antigen dispersions from all tube were pooled and then divided into five equal parts by weight so as to contain 20 mg of the CFA/II each. The desalted antigen dispersion was stored at -10 to -20° C.

Freeze Drying of the Desalted CFA/II Dispersion. 80 mg of sucrose was added to each part of the CFA/II dispersion. The resulting mixture was flash-frozen using a dry ice-acetone bath (100-150 ml od acetone and 50-100 g of dry ice). The frozen solution was freeze dried overnight using Repp Sublimator 16 freeze dryer at vacuum of 1 micrometer of mercury and a shelf temperature not exceeding 37° C.

CFA/II Biodegradable Polymer Microspheres.

Particle size distribution. About 1 mgm of microspheres were dispersed in 2 ml of 1% Polysorbate 60® (Ruger Chemical Co. Inc. Irvington, N.J.) in water in a 5 ml capacity glass vial by sonication. This dispersion was observed under a calibrated optical microscope with 43x magification. Using a precalibrated eye-piece micrometer, the diameter of 150 randomly chosen microspheres, was determined and the microsphere size distribution was determined

Scanning Electron Microscopic Analysis.

Microspheres were sprinkled or the surface of 10mm stub covered with a non-conductive adhesive (Sticky-Tab, Ernest F. Fullem, Inc., Lutham, N.Y.) Samples were coated with gold/palladium in an automatic sputter-coating opparatus (Samsputter-2A, Tonsimis Research Corporation). The samples were examined with a Hitachi S-450 scanning electron microscope operated at 15-20 KV.

Preparation of CFA/II Microspheres. Solvent extraction techique was used to encapsulate the freeze dried CFA/II into poly(lactide-co-glycolide)(Medisorb Techologies International, visocity 0.73 dl/g) microspheres in the 1-10 um size range to achieve theoretical antigen loading of 1% by weight. The freeze dried antigen-sugar & matrix was dispersed in an acetolnitrile solution of the polymer and then emulsified to achieve desired droplet size. Microspheres were solidified and recovered by using heptane as extracting solvent. The microsphere batches were pooled and vacuum dried to remove traces of solvent.

Protein Content. The CFA/II microspheres were dissolved in 0.9% SDS in 0.1N NaOH for 18 hr with stirring then neutralized to pH 7 and assayed. The micro bicichoninic acid (BCA) method was utilized with

both lactic acid and glycolic acid blanks and compared to bovine serum albumin (BSA) standard and results expressed as percent by weight.

Moisture Content. One hundred and fifty mgm of CFA/II microspheres were dissolved in 3 ml of acetonitrile by sonication for 3 hours. One ml sample was injected into a Karl Ficher titrimeter and triter reading observed was recorded and acetonitrile blank was substracted to determined percent water content.

Acetonitrile and Heptane Residuals. Ten mgm of CFA II microspheres were dissolved in 1 ml DMF then analysed using gas chromatography and comparing peak heights to external standards of either acetonrile or heptane diluted in DMF with 10 mgm of blank microspheres. The results are expressed as percent by weight.

Microbial load. One hundred mgm of CFA/II microsphere(single dose) are suspended in 2 ml of sterile saline than poured into 2 blood agar plates (1 ml each). All colonies are counted and identified after 48 hours in culture at 37° C and expressed as total number. Similiar amount of microspheres is in 0.25 ml aliquots poured onto 4 different fungal culture plates (Sabhiragar, casein peptone agar with chloramphenicol, brain heart infusion agar with

chloramphenol and genimycin or chloramphenicol alone) and cultured at 30° for 5 weeks and the colories counted & identified and expressed as total number.

CFA/II Release From Microsphere Study. Thirty mgm samples in triplicate were placed in 2 ml conical upright microcentrifuge tubes containing 1 ml of PBS at pH 7.4. The tubes were capped and kept immerized in a water bath maintained at 37° C with constant agitation. The samples were withdrawn at 1, 3, 6, 8, 15 and 22 hour time intervals by centrifuging the sample tubes for 5 minutes at the maximum speed of bench top centrifuge. The release medium was collected through a 5 um nylor screen for CFA/II protein analysis using the micro BCA method and comparing results to BSA standard and expressing results as percent cumulative release of CFA/II.

General Safety Test. Two doses of one hundred mgm CFA/II microspheres were suspended by sonication for 5 minutes in 3.1 mls of sterile vaccine dilutent consisting of injectable saline containing 0.5% Polysorbate 60^{R} N.F., 0.03 ml were injected intraperitoneally into each of 2 mice and 3 mls were administered by gastric lavage to each of 2 guinea pigs. The animals were weighed both before and at 7 days following the vaccine administration. All animals were observed daily for any signs of toxicity.

Rabbits. 1.5-2 kilogram male specific pathogen free New Zealand white rabbits, obtained from closed colony maintained at the National Institute of Health, Bethesda, MD. They were selected for study if they did not have measurable serum antibodies at 1:2 dilution to CFA/II antigens by ELISA and were not colonized by E. coli as determined by culture of rectal swabs.

Intra-Muscular Immunization of Rabbits and ELISA. Two Rabbits were immunized with CFA/II microsphere vaccine at 25 ug protein in two different sites intra-muscularly on day 0. Sera were obtained from all animals before immunization on day o and days 7 and 14. The sera were tested by ELISA for IgG antibodies to CFA/II antigen and individual coli surface (CS) proteins CS3 and CS1. ELISA plates were coated with 3 ug/ml of either CFA/II antigen, CS3 or CS1 protein (150 ul/well) and incubated with 150 ul/well of PBS with 0.1% BSA for four hours at room temperature. The PBS with 0.1% BSA is washed out with PBS and 100 ul/well of different dilutions of each rabbit serum in triplicate was added to the plates. The dilutions ranged from undiluted to 1:1,000,00. The plates were incubated with the sera for 3 hours at 37° C. The sera were washed out with PBS and then horse radish peroxidase-conjugated goat anti- rabbit IgG was added to the plates at a 1:1000 dilution (100 ul/well). The plates

were incubated for 1 hour at room temperature with the peroxidase conjugate. The conjugates were washed out of the plates with PBS and 100 ul/well of an ABTS substrate solution (Kikegaard and Perry Laboratories) was added to each well in the plates. The plates were read using the ELISA reader(Dynatech Laboratories MR 580) at a wave length of 405 nm after 15 minutes. The results are measured and expressed as antibody titers.

Intra-duodenal Vaccination of Rabbits. Rabbits (N=5) were vaccinated with CFA/II microspheres containing either 25 or 50 ug of protein suspended in 1 ml of PBS containing 0.5% Polysorbrate 60® on day 0 and 7 by sonication. The microspheres were injected through an Olympus BF type P10 bronchoscope into the duodenum of the rabbits following sedation with an intra muscular injection of ketamine HCl (50 mgm I.M.)(Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) and Lylazine (10 mgm I.M.) (Rompom Malay Corporation, Shnanee, KS). The endoscope was advanced ready under direct vission into the stomach which was insufflated with a 50 ml bolus of room air via a catheter passed through the biospy channel. The catheter was advanced through the pylorus 3-4 cm into the duodemum and the microsphere suspension in 1 ml of PBS was injected, followed by a 9 ml flush of PBS and removal of the air

bolus. The rabbits were sacrified by chemical euthanasia at day 14.

Anti-CFA/II Stimulated Lymphocyted Transformation. The Peyer's Patchs were removed and cell suspension obtained by teasing and irigation with a 20 guage needle and syringe. The cells were placed in 2 ml of media at a concentration of 2.5 x 10^6 cells/ml for each well of a 24 well plate. These cells were challenged separately with BSA and the CFA/II antigen at doses of 500, 50 and 5 ng/ml in triplicate wells. The plates were incubated at 37° C with 5% CO_2. On day 4 the cells were mixed while still inside the wells and 100 ul were transferred into each of 4 wells in a 96 well flat bottom microculture plate. Thus, the challenge at each antigen dose represented by 3 wells in the 24 well plate is now represented by 12 wells in the 96 well plate. After the cells have been transferred, each well is pulsed with 20ul of 50 uCi/ml tritiated thymidine. These pulsed plates were incubated for 6 hrs then harvester with 96 Mach II Cell harvested (Tourtec, Inc.). The lymphocyte proliferation was determined by the tritriated thymidine incoporation measured in kilo counts per minute (Kcpm) using the 1205 Beta Plate Liquid scintillation counter (LKB, Wallac, Inc.). The results

are expressed as mean Kcpm ± SD and compared to media controls.

Anti-CFA/II Antibody Secreting B Cells. Spleen cells were obtained from immunized rabbits on day 14 following intra-duodenal immunization with CFA/II microsphere vaccine. The cells were placed in 96 well round bottom microculture plate at a final concentration of 6 x 10^5 cells/well and incubated for 0, 1, 2, 3, 4 and 5 days at 37° C with 5 CO_2. 96 well flat bottom microculture plates were coated with 3 ug/ml of CFA/II antigen overnight blocked with PBS with 0.05% Polysorbate 60®. On the harvest days, the cells were gently flushed out of the wells of the round bottom plates and transferred to the corresponding well in the antigen coated, 96 well flat bottom microculture plates to be tested for the presence of antibody secreting cells using ELISPOT technique. The plates were incubated with the cells overnight at 4° C. The cells were then washed out of the flat bottom plates with PBS, and 100 ul/well of horserudish-peroxidase conjugated, goat anti-rabbit total antibody (IgM, IgG, and IgA) at a 1:1000 dilution were added to the plates. The Plates were incubated for 1 hour at room temperature, at which time, the conjugate was washed out of the plates with PBS. 0.1 mgm of agarose was dissolved in 10 ml of PBS by boiling. After the agar

solution cooled but not hardened, 6 mgm of 4-chloro-naphthol, 2 mls of methanol and 30 ul of hydrogen peroxide were added to make the substrate solution. The solution was placed into the flat bottom plates (100 ul/well) and the plates were held at 4°C overnight so the agar could harden. The number of browish spots per 15 wells (total of 9 x 10^6 spleen cells) was counted and represents the number of antibody secreting cells per 9 x 10^6 spleen cells.

Pathological Evaluation. Rabbits were euthanized by parenteral overdose of sodium pentobarbital and were subjected to complete necropsy. Sample of tissue including small and large intestine with gut associated lymphoid tissue, spleen, mesenteric and mediastinal lymph nodes, lung, trachea, liver and kidney were fixed by immersion in 10% neutral buffered formalin. Tissues were routinely processed for light microscopy and embedded in paraffin. Five micron thick sections were stained with hematoxylin and eosin.

Statistical Analysis. The paired student t-test was used to determine p values.

RESULTS

Particle Size Distribution. The results of size frequency analysis of 150 randomly chosen microspheres are shown in (Figure 29). The particle size

distribution is plotted in % frequency against particle size in diameter (size) expressed in um. The average number fregency diameter is 4.6 um. The average volume frequency diameter is 4.6 um. The percent volume between diameters of 5-10 um is 63% and the percent volume less than 10um diameter is 88%.

Scanning Electoron Microscopy. The microspheres are seen in (Figure 30) which is a scanning electron photomicrograph. Nearly all the microspheres are less than 10 um as compared to the 5 um bar. Also the surfaces of the microsphere are smooth and demonstrate lack of pores.

Protein Content. The protein loads of the individual batches are the following: K62A8, 1.16% ± 0.10 SD; K63A8, 1.023% ± 0.17SD; K64A8, 1.232% ± 0.13 SD; and K65A8, 0.966% ± 0.128 SD. The mean average protein load is 1.16% ± 0.15 SD. The protein load of the CFA/II microsphere vaccine in the final dose vial is the following: Lot L74F2, 1.175% ± 0.17SD.

Moisture Content. The CFA/II microsphere vaccine (Lot 74F2) percent water content was found using the Karl Fischer titrimeter method to be 2.154% using triplicate samples.

Acetonitrile and Heptane Residuals. The acetonitrile residuals of the 4 individual CFA/II microsphere batches are the following: K62A8, <0.1%;

K62A8, <0.1%, K64A8, <0.1%; and K65A8, <0.1%. The acetonitrile residual of the CFA/II microsphere vaccine in the final dose vial is the following: Lot L74F2, 0.07 ± 0.05%. The heptane residual of the 4 individual CFA/II microsphere batches are the following:K62A8, 1.9%; K63A8, 1.4%; K64A8, 1.6% and K65A8, 1.6%. Following pooling in heptane and subsequent drying, the heptane residual of the CFA/II microsphere vaccine in the final dose vial is the following: Lot L74F2, 1.6 ± 0.1%.

Microbial load. One hundred milligrams (a single dose) of CFA/II microsphere vaccine (Lot L74F2) in the final dose vial was suspended in a 2 ml of sterile saline and 1 ml poured onto a blood agar culture plate x 2. Twenty two colonies grew after 48 hours of culture and 21 were identified as coagulase negative staphlycoccus and 1 as a micrococus species. All these bacteria are considered to be nonpathogenic to humans. An additional 100 mgms of CFA/II microsphere vaccine (Lot L74F2) were suspended in 2 ml of sterile saline and 0.25 ml poured onto four different fungal culture agars and cultered for 5 weeks. Three fungal colonies grew and each was identified as A. glaucus.

CFA Release From Microsphere Study. Three thirty mgm samples were incubated each in 1 ml of PBS, pH 7.4

at 37° C for 0, 1, 3, 6, 8, 15 and 22 hours. The superanates were removed and replaced at these times. The protein content was determined for each supernate sample and the results are seen in (Figure # 31). The results are plotted as percent release of CFA/II against time in hours. An average of 8% of CFA/II is released at one hour rising to 20% at 8 hours then a slower release to 25% at 22 hours.

General Safety Test. Two one hundred milligrams (a single dose) of CFA/II microsphere vaccine in the final dose vials were suspended in 3.1 mls of the sterile dilulent consisting of 0.85 N saline prepared for injection plus Polysorbrate 60® at 0.5%. Two Swiss mice (16.5 gm) were injected intraperitoneally with 0.03 mls and two Hartley guinea pigs (350 gm) were administered by gastric lavage 3.0 mls.

None of these animals displayed any signs of toxicity for 7 days. The mice gained and average of 2.3 gms and the guinea pigs gained and average, of 43 grams. The CFA/II microsphere vacccine therefore passed the general safety test.

Serum IgG Antibody Responses. Two rabbits were immunized in two separate sites intra-muscularly with 25 ug of protein of CFA/II microsphere vaccine (Lot L74F2) in the final dose vial. Sera samples were obtained before and 7 and 14 days following

immunization. The IgG antibody titers to CFA/II CSI and CS3 protein were determined using ELISA and the results seen in (Figure 32). The results are expressed as mean antibody titers against the different antigens at 0, 7 and 14 days. High antibody titers greater than 1000 were seen at 7 days to both CS1 and CS3 protein which rose to greater than 10,000 by day 14. The individuals titers to CFA/II are seen in (Figure 33). Rabbit 109 developed an antibody titer of 1,000 by day 7 rising to 3,000 by day 14. Rabbit 108 had a log higher rise at day 7 and 2 log higher rise at day 14 being 3×10^4 at day 7 going to 1×10^5 at day 14.

Anti-CFA/II Stimulated Lymphocyte Transformation. Five rabbits were immunized intra-duodenally with CFA/II microspheres containing either 25 ug of protein (human dose equivalent) or 50 ug of protein on days 0 and 7 and then sacrificied on day 14. The Peyer's patch lymphocytes were challenged in vitro with CFA/II antigen, BSA media and alone. The lymphocyte transformation was determined by tritriated thymidine incorporation. The results of the high dose immunization are seen in (Figure 34). The results are expressed as Kcpm against antigen dose. No response to BSA or media control is seen in any of the five rabbits. All rabbits responded by lymphocyte

transformation in a dose dependent manner to the CFA/II.

The highest dose responses were 3-10X's the media control are highly significant with a p value of <0.002. The results of the 5 rabbits receiving the low dose immunization are seen in (Figures 35). Rabbit #80 gave no response probably due to poor Peyer's patch cell population which did not respond were to Conconavallin A mitogenic stimulation either. The remaining 4 rabbits gave positive responses with the high CFA/II dose response being 2-8x media control and highly significant with p values of <0.009. Again no response were seen to BSA compared to the media cont

Anti-CFA/II Antibody Secreting B-Cells Five rabbits immunized intraduodenally with CFA/II microsphere containing 50 ug of CFA/II protein at days 0, 7 than sacrified at day 14 were studied. The spleen cells were placed into microculture then ELISPOT forming B-Cells secreting specific anti CFA/II antibody determined at days 0, 1, 2, 3, 4 and 5. The results are seen in (Figure 36) and expressed as # of antibody secreting cells per 9 x 10^6 spleen cell against culture days. Positive responses were seen in all 5 rabbits on days 2-5. Days of maximum responses occurred on day 3 for rabbits 65 and 66; day 4 for rabbit 85; amd day 5 for rabbits 83 and 86. The responses are highly

significant being 7-115 times higher than the 1-2 cells seen on all days in 4 control rabbit (67, 69, 72, 89) (Figure 37). Here is a composite graph expressing the mean counts ± ISD for all days of culture.

Pathological Evaluation. A consistent finding in the spleens of all rabbits both the 25 and 50 ug protein dose groups was minimal to mild diffuse lymphocytic hyperplasia the periarteriolar lymphatic sheaths (T cell dependent areas). Two of five rabbits of the 50 ug dose group (#83 and #86) also had mild lymphocytic hyperplasia of splenic follicular (B cell dependent) areas. The three rabbits in an untreated control group had histologically normal spleens.

Reactive hyperplasia of mesenteric lymph nodes was often seen in vaccinated rabbits. Two of five rabbits in the 25 ug dose equivalent group (#83 and #86) also had minimal to mild lymphocytic hyperplasia of cortical follicular (B cell dependent) areas. The mesenteric lymph nodes of the other vaccinated rabbits and of the untreated control rabbits were within normal limits. Incidental or background lesions found in one or more rabbits of all three group were acute minimal to mild pnuemonia and foreign body microgranulomas of the cecal gut associated lymphoid tissue.

Disscussion

McQueen et al (33)has found that the AF/R1 adhesin of rabbit diarrheagenic Escherichai coli (RDEC-1) incorporated into biodegrable microspheres could function as a safe and effective oral intestinal vaccine in the rabbit diarrhea model. The AF/R1 was incorporated into poly D,L-lactide-co-glycolide) microspheres and administered intraduodenally. Jarboe et al (34) reported that

Peyer's patch cells obtained from rabbits immunized intra-duodenually with AF/R1 in microspheres responded with lymphocte proliferation upon in vitro challenge with AF/R1. This early response at 14 days gave a clear indication as to the immunogenicity of E. coli pili contained within the polymer microspheres.

In developing an effective oral vaccine against enterotoxigenic E. coli, CFA/II pili given as an oral vaccine was found to be ineffective. The CFA/II pilus proteins were found to be rapidly degraded when treated with 0.1mHCl and pepsin conditions mimicking those contained in the stomach (27). The CFA/II was found to be immunogenic when given in high doses intra-intestinally producing intestinal secretary IgA antibodies (26).

The CFA/II vaccine has now been incorporated into poly(D,L lactide-co-glycolide) microspheres under Good Manufacturing Practices and tested under Good

Laboratory Practices. The microspheres, are spherical, smooth surfaced and without pores. The majority (63%) are between 5-10 um in diameter by volume. This size range has been suggested to promote localization within the Peyer's patch in mice and perhaps enhance local immunization (29-32). The protein content being 1.174% is close to 1% which was the goal of the vaccine formulation. One percent was chosen because 0.62% was the core loading of the AF/R1 microspheres which were effective. Also a small precentage perhaps 1-5% (35) is anticipated to be taken up from the intestine, a higher protein content would lead to considerable loss of protein.

The organic residuals are of course a concern. Heptane exposure would be 1.7 mgm per vaccine dose. This is compared to the occupational maximum allowable exposure of 1800 mgm/15 min. Therefore, the heptane contained with the CFA/II microsphere vaccine appears to be a safe level. The acetonitrile is very low - 0.1 mgm per vaccine dose. The human oral TDLO is 570 mgm\Kg (any non letheal toxicity). Therefore, the acetonitrile contained with the CFA/II microsphere vaccine appears to be at a safe level. The CFA/II vaccine was produced under sterile conditions. However, the process of incorporation of the desalted CFA/II vaccine into the polymer microsphere batches and

subsequent pooling and loading final dose vials was done in a clean room as for any oral medication. It was expected and found that there was be a microbial load. The guide used was the World Health Organization (WHO) Requirements of Thyphoid Vaccine (Live Atttaruated, Ty 21a oral). Two hundred non pathogenic bacteria are allowed as well as 20 fungi per dose. The CFA/II microsphere vaccine is well under these requirements having only 22 non-pathogenic bacteria and 3 fungi per dose.

The general safety test was also patterned after the WHO requiremets for the TY, 21a oral vaccine in that the CFA/II microsphere vaccine was give by gastric lavage to the guinea pigs. Both mice and both guinea pigs demonstrated no toxicity & gained weight over the 7 day test clearly indiciating the innoccuos nature of this vaccine by passing this safety test.

The CFA/II microsphere vaccine (Lot74F2) is immunogenic giving high titer serum IgG antibody responses as early as 7 days following intra muscular injection in rabbits. This test will be used as potency test for future lots of the CFA/II microsphere vaccine. Slighly higher antibody titers were seen towards the CS3 pilus protein and this may reflect that CS3 accounts for 90% of the protein in the CFA/II and CS1 10% (36).

The CFA/II microsphere vaccine was also immunogenic following intra-duodenal administration to rabbits. The highest lymphocyte proliferative responses from Peyer's patch cells were seen with the lower 25 ug dose. This is the human equivalent dose and suggests that higher doses of antigen in polymer microspheres may attenuate, this immunological reponse.

The antibody secreting B-cells demonstrated in the rabbit spleen at 14 days is a clear indication that B-cells have been immunized. They may represent resident B-cells immunized in the spleen or B-cells immunized at the level of the Peyer's patches and are migrating through the spleen to return to the intestial mucosal lamina propria (1-3). The delay of several days before secreted antibody is detected suggests either manuration is required of the B-cells or that down regulation may be present initially and lost with time in culture.

Further evidence of immunization by the CFA/II microsphere vaccine given intra-duodenually is demonstrated by the lymphatic hyperplasia in the spleen seen to a greater extend in the rabbits receiving the lower dose 5/5 compared to 2/5 of the rabbits receiving the higher 50 ug protein dose. On the other hand, greater T-cell dependent area lymphoytic hyperplasia in the mesenteric lymph nodes were seen in rabbits

receiving the higher 50 ug dose, 4/5 compared to 2/5. These changes are most likely due to the vaccine since similar changes were not seen in three untreated control rabbits. Also no abnormal pathological changes attributable to the vaccine were seen.

The CFA/II BPM vaccine has undergone pre-clinical evaluation and has been found safe and immunogenic. This vaccine is ready for clinical Phase I safety testing following FDA's IND approval.

PHASE IV

In sum, alum precipitation, vaccination regimen and controlled delivery by microencapsulation were studied to determine what criteria must be satisfied to provide a protective immune response to hepatitis B surface antigen (HBsAg) after a single injection of vaccine. In mouse studies, the 50% effective dose (ED_{50}) for the alum precipitated Heptavax B vaccine (Merck, Sharp and Dohme) was 3.8 ng when administered in a 3 injection regimen, but was 130 ng when one immunizing dose was used. Antigen release studies revealed that HBsAg is bound tightly to the alum, indicating that the antigen remains in situ until scavenged by phagocytic cells. the ED_{50} with a 3 dose regimen of aqueous HBsAg was 180 ng, a opposed to over 2000 ng for daily injections of low doses for 90 days and 240 ng for a regimen that employed initially high

doses that decreased geometrically at 3 day intervals over 90 days. The ED_{50} was 220 ng for a single dose regimen of HBsAg microencapsulated in poly (DL-lactide-co-glycolide) in a form that was too large to be phagocytized and had an antigen release profile similar to that achieved with the geometrically decreasing regimen of doses. This indicates that single injection of microencapsulated immunogens can achieve similar effects in vivo to those achieved with multiple dose regimens. For HBsAg the effect to be achieved appears to be 3 pulses of particulate immunogens that can be scavenged by phagocytes.

INTRODUCTION

A major disadvantage of inactivated vaccines lies in their inability to confer lasting immunity. Due to rapid elimination from the body, multiple doses and boosters are usually required for continued protection[37]. Alum adjuvants, achieving their effects by mechanisms of antigen presentation and sustained antigen release[38], have been used successfully to increase the potency of several inactivated vaccines including those against tetanus, anthrax, and serum hepatitis[39,40]. Though useful, alum preparations are deficient in several aspects. Control over quantity and rate of antigen release is limited, often resulting in a continued requirement for immunization schedules

consisting of multiple injections given over a period of several months to years. Alum adjuvants are also non-biodegradable and thus remain within the body, serving as a nidus for scar tissue formation[38] long after they have served their function.

Protracted, multiple immunization schedules are unacceptable during massive mobilization and deployment of troops. Changing global disease patterns and deployment of new biological warfare agents by enemy forces require flexibility in the number and types of vaccine antigen administered to soldiers departing for combat. Any immunization schedule requiring completion during engagement in non-linear combat would compromise this flexibility and place an unreasonable burden on our health care delivery system.

The main objective of this study was, therefore, to develop a biodegradable, controlled-release adjuvant system capable of eliminating the need for multistep vaccination schedules. This investigation was designed to : (1) determine in an animal model hepatitis B vaccine release rate characteristics desirable for single-step immunization, (2) incorporate those release rate characteristics into a one-step biodegradable poly(DL-lactide-co-glycolide)(DL-PLG) microencapsulated hepatitis B surface antigen (HBsAg) vaccine, and (3) conduct an <u>in vivo</u> trial comparing the effectiveness of

this single-step vaccine against the conventional three-step hepatitis vaccine currently employed[41]. The results were intended to provide the foundation for further development of single step vaccines against hepatitis and other militarily significant diseases[42].

MATERIALS AND METHODS

Vaccine potency assay. Due to its availability, compatibility with cage mates, and potential application to the study of hepatitis B vaccine[43], the female Walter Reed (ICR) stain mouse was used. A hepatitis B vaccine potency assay for comparing the six-month immunization schedule currently in use[41] with that of a single-step immunization by sustained antigen release was established according to the following protocol: Specimens for baseline antibody titers were collected from twenty mice by exsanguination. Immediately prior to exsanguination, all mice employed in this and other exsanguination procedures in these studies were anesthetized with a 0.1 ml injection of V-Pento. Groups of 12 mice were then immunized according to a schedule consisting of either 0.25 ug, 0.025 ug, 2.5 ng, 0.25 ng, 2.5 pg, or 0.25 pg Heptavax B vaccine (HBV) administered in 50 microliter volumes subcutaneously (s.c.) at the beginning and end of the first, and end of the second month of the protocol. Antibody responses to the vaccine were monitored

immediately before the third injection and approximately one month after the third injection. Specimens for antibody determination were collected by exsanguination of seven anesthetized mice from each group and assayed along with the baseline samples by the Abbott Ausab radioimmunoassay. Percent seroconversion verses micrograms vaccine employed with calculated by the method of Reed and Muench[43]. These data were employed to establish a mouse vaccine potency assay calibrated to detect differences between Heptavax B and other forms of hepatitis b vaccine.

In vitro antigen release rate from Heptavax B vaccine. Antigen release from aluminum hydroxide adjuvant in HBV was measured by pumping 2 cc per hour of 1:20,000 thimerosal in saline at 4°c across a 0.2 u pore diameter Acrodisc filter apparatus containing 20 ug of vaccine. The effluent, collected by a Gilford fraction collector, was assayed periodically over several weeks for protein by UV absorption at 280 nm on a Beckman model 25 double beam spectrophotometer, and for HBsAg by the Abbot Ausria II radioimmunoassay made quantitative by using HBsAg standards supplied by Merk, Sharp, and Dohme. Accuracy of the HBsAg standards were verified by Biuret protein determination and by UV absorbance at 215 nm and 225 nm[44]. Nonspecific antigen retention on the Acrodisc filter was assessed by

measuring percent recovery of a known quantity of HBsAg. Spontaneous degradation of vaccine antigen was monitored by comparing daily rations of antigen to total protein detected in the effluent.

Evaluation of HBsAg stability. These studies were designed to characterize the stability of the aqueous antigen to the various physical conditions employed in the microencapsulation process. Conditions tested included lyophilization with reconstitution in distilled water, cyclohexane, methylene chloride, chloroform, methyl alcohol, acetone, iso-octane, hexane, acetone, pentane, or heptane; irradiation while lyophilized; and, exposure to elevated temperatures. Samples exposed to organic solvents were first lyophilized, reconstituted with the test solvent, evaporated to dryness under nitrogen at room temperature and reconstituted with distilled water. Test samples were compared against untreated controls by assaying serial dilutions of each with the Abbot Ausria II procedure and comparing the plots of counts per minute verses dilution.

Assessment of the effect of antigen release rate on vaccine potency. Three regimens simulating patterns of free HBsAg release that could be achieved by microencapsulation were contrasted with the three monthly dose regimen of Heptavax B for immunizing mice.

To do so, 24 ICR mice were divided into groups and vaccinated as indicated below. Seven mice from each subgroup were exsanguinated at the end of the second and third months of the experiment. The sera were separated and assayed for specific antibody response to HBsAg by Abbot Ausab procedure.

HV regimen a: 14 mice/treatment receiving 3 s.c. injections of 250, 25, 2.5 or 0.25 ng doses of HBV a month apart.

HBsAg regimen a: 14 mice/treatment receiving 3 s.c. injections of 250, 25, 2.5 or 0.25 ng doses of aqueous HBsAg a month apart.

HBsAg regimen b: 14 mice/treatment receiving total doses of 750, 75, 7.5 or 0.75 ng of aqueous HBsAg over 3 months by s.c. injections of ZX_Y ng at 3 day intervals, where Z is the total dose, y is the injection number, and X is the fraction indicated on the graph in Fig. 1 minus the fraction for the previous injection.

HBsAg regimen c: 14 mice/treatment receiving daily s.c. injections of 8.33, 0.833, 0.0833 or 0.00833 ng of aqueous HBsAg for 3 months.

Microencapsulation in DL:PLG. Microencapsulated immunogens were fabricated by Southern Research Institute, Birmingham, AL. DL-PLG polymers were synthesized from the cyclic diesters, DL lactide and

glycolide, by using a ring-opening melt polymerization catalyzed by tetraphenyl tin[45]. The resulting polymer was dissolved i methylene chloride, filtered free of insoluble contaminants and precipitated in methanol. Lactide-co-glycolide mole ration of the product was determined by nuclear magnetic resonance spectroscopy. Encapsulation of HBsAg in DL:PLG polymer was achieved by an organic phase separation process[46]. Microcapsules of the desired size (approximately 100 micron diameter in these studies) were isolated from each batch by wet sieving with hexane through standard mesh stainless steel sieves and then dried for 24 hours in a vacuum chamber maintained at room temperature.

In vitro analysis of encapsulated antigens. Integrity of encapsulated antigen was assessed by comparing the antigen to total protein ratios present in microcapsule hydrolysates with those obtained from suspensions of pure unencapsulated antigen. Centrifuge tubes containing 1 ug of either microencapsulated or pure vaccine antigen in 1 ml saline were incubated at 4_{o}c with shaking. Samples were collected at weekly intervals by interrupting the incubation, sedimenting the contents of the tubes by centrifugation and withdrawing the supernates. Sediments were resuspended in 200 microliters of saline and supernates were assayed for HBsAg by the Abbott Ausria II

radioimmunoassay. The HBsAg standard described earlier in this report was used as the calibrator. Antigen destruction due to the encapsulation procedure was monitored by a comparison between the antigen assayed from the hydrolysate and from the untreated antigen control.

Assessment of the potency of DL:PLG microencapsulated HBsAg for immunizing ICR mice when used alone and in combination with Heptavax B vaccine. HBsAg loaded microcapsules that had been fabricated by Southern Research Institute to release the majority of their HBsAg load within 40 to 50 days were serially diluted in 10-fold steps by mixing the dry, loaded capsules with blank placebo capsules of similar size and composition. The resulting stock and diluted microcapsule preparations were placed onto lyophilizer when not in use in order to assure minimum spontaneous degradation prior to injection. On the day of injection, a predetermined weight of microcapsules or placebo-diluted microcapsules was added to each syringe. Immediately prior to injection either one or two ml of injection vehicle (2 wt % carboxymethyl cellulose and 1 wt & Tween 240 in water, Southern Research Institute) were drawn into the microcapsule-loaded syringes, mixed and injected. All mice were vaccinated s.c. as indicated below:

Group 1: 14 mice/treatment receiving 25, 25, 2.5, 0.25 or 0.925 ng HBV.

Group 2: 14 mice/treatment receiving 1000, 250, 25 or 2.5 ng aqueous HBsAg with Bovine Serum Albumin (BSA).

Group 3: 7 mice receiving 1600 ng microencapsulated HBsAg (HBsAg) plus 0.25 ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or 0.16 ng HBsAg plus 0.25 ng HBV.

Group 4: 7 mice receiving 1600 ng HBsAg plus 2.5 ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or 0.16 ng HBsAg plus 2.5 ng HBV.

Group 5.: 7 mice receiving 1600 ng HBsAg plus 25 ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or 0.16 ng HBsAg plus 25 ng NBV.

Group 6: 7 mice receiving 2500 ng HBsAg and 14 mice-treatment receiving 250, 25, 2.5 or 0.25 ng HBsAg. Fifty-three days after receiving the above injections, the mice were anesthetized with an 0.1 cc injection of V-Pento and exsanguinated. Blood samples were allowed clot and the sera were separated by centrifugation. The serum samples were assayed for antibody to HBsAg by the Abbott Ausab procedure.

RESULTS

Heptavax B vaccine potency. As can be seen from Table 4, the total dose of vaccine which produced seroconversion in 50% of

TABLE 4. Potency of Heptavax B vaccine in ICR mice.

No. Inj.	ng Heptavax B per Injection						
ED_{50} ng	250	25	2.5	.25	.025	.0025	.00025
2 1.7	5/5	4/4	3/6	2/6	0/5	1/4	0/4
3 2.0	6/6	6/6	4/6	1/6	0/6	1/6	1/6

* Number positive seroconversions per number vaccinated.

The vaccinated mice (ED_{50}) for HBV was approximately 2 ng, whether the vaccine was given in 2 or 3 injections.

In vitro antigen release rate from HBV. HBsAg release from the 20 ug of Heptavax was not detected in any of the 21 fractions of saline collected from the Acrodisc polycarbonate filter over a 30 day period. The lower limit of detection for the Abbott Auria II assay employed was approximately 4.8 ng/ml. The

Acrodisc filter used in the antigen release study was back-washed with 10 mls normal saline. Quantitation of the HBsAg present within this back-wash eluent revealed the presence of the original 40 ug of Heptavax vaccine which had been loaded into the filter at the start of the experiment. This is the concentration which one would expect to obtain if there had been no deterioration of the original 40 ug/ml HBsAg loaded onto the filter, none of the antigen eluted from the alum adjuvant, and none of the vaccine had adsorbed onto or passed through the filter.

Evaluation of antigen stability. Considerable effort was expended in assessing the effects of physical conditions on the antigenicity of HBsAg to insure that the conditions used for microencapsulation would not cause serious degradation of the immunogen. Since microencapsulation must be performed on dried materials which are suspended in organic solvents, the HBsAg, which was provided as a solution, had to be lyophilized. Initial attempts at lyophilizing HBsAg in normal saline resulted in a total loss of detectable antigen within samples. Dilution of the HBsAg sample 1:10 in distilled water prior to freezing resulted in reservation of nearly 100% of the antigen detectable in the original sample. Studies of antigen stability at elevated temperature revealed that HBsAg may be heated

to 50°c for up to one hour without appreciable loss of antigen. The studies involving exposure of lyophilized antigen to organic solvents indicated that iso-cane and hexane had minimal effects on antigenicity, but that 95% to 100% of antigenicity was lost upon exposure to either methylene chloride, chloroform, cyclohexane, or methyl alcohol. Moderate antigen loss occurred in the presence of acetone, pentane and heptane. As a result of these studies, hexane was chosen as the solvent for microencapsulation.

Assessment of the effect of antigen release rate on vaccine potency. The results (Table 5) indicated that immunogen formation (i.e., the alum adjuvant of Heptavax B) had far more

TABLE 5. Effect of immunogen formulation and vaccination regimen on potency for immunizing ICR mice.

Immunogen Formulation	Regiment	ng Total Dose HBsAg 750	75	7.5	.75	ED_{50} ng
Heptavax B	a	7/7*	6/6	5/7	1/7	3.8
Aqu. HBsAg	a	4/6	3/7	0/7	0/6	180
Aqu. HBsAg	b	6/7	0/7	1/7	0/7	240
Aqu. HBsAg	c	1/7	0/7	0/7	0/7	>2000

* Number positive seroconversions per number vaccinated.

a 3 injections of 1/3 total dose a month apart.

b Injections administered every three days for 90 days in decreasing dosages according to a logarithmic progression.

c Injections of 1/90 total dose daily for 90 days.

effect on potency than did the vaccination regimen, and that pulsing with large doses of immunogen was more effective than continuous administration of small doses.

HBsAg release from DL:PLG microcapsules. The microcapsules employed in this study were designed to

disintegrate within three weeks after hydration. It is evident from the release curve (Fig. 2) that they performed as designed, releasing approximately 17% of their total load in an initial pulse and approximately 7% of the remaining available HBsAg over the first three weeks.

Assessment of the potency of DL:PLG microencapsulated HBsAg for immunizing ICR mice when used alone and in combination with Heptavax B vaccine. The results (Table 6) indicate that the microencapsulated HBsAg had approximately the same immunogenicity as did the Heptavax B. Neither immunogens were sufficiently potent to effect with a singly injection seroconversion rates similar to those achieved after three injections of Heptavax B (Table 4). Only the immunogen

TABLE 6. Potencies of Heptavax B and microencapsulated HBsAg by single injection S.C. when administered alone and in combination to immunize ICR mice.

Var. Dose ng Const.	ng Variable Dose				Var. Dose Tot. Dose
Immunogen Dose mHBsAg	2500	250	25	2.5	.25 ED_{50} ng ED_{50} ng

Heptavax B	0	13/14*	8/14	4/14	0/13		130
130							
Heptavax B	0.16		11/13	4/14	1/14		1.7
1.8							
Heptavax B	1.6		10/13	1/14	0/13		100
100							
Heptavax B	16		3/14	1/14	1/14		>470
>490							
Heptavax B	160		3/12	2/11	1/12		>370
>530							
Heptavax B	1600		7/7	7/7	7/7		<0.8
1600							
Mic. HBsAg	0	3/6	6/15	1/13	2/10	2/14	220
220							

* Number positive seroconversions per number vaccinated.

combination of Heptavax B with 0.16 ng mHGsAg provided this level of seroconversion. At the ED_{50} endpoint, the 0.16 ng dose of mHGsAg is approximately 10% of the total dose. Similarly, a small amount of Heptavax B appeared to enhance the immunogenicity of the microencapsulated immunogen, although the combination was clearly less immunogenic when the two formulations were present at equivalent concentrations.

TABLE 7. ANTIBODY SECRETING CELL RESPONSES TO CFA/II VACCINE BY ELISPOT ASSAY AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28 (E. COLI CVD 15001)

Vaccinee	IgA					IgG					IgM				
	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35
15001-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-3[1]	0	22	0	0	10	0	0	0	0	0	0	0	0	0	0
15001-4	0	6	0	0	16	0	0	0	0	4	0	6	0	0	0
15001-6[2]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-8	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
15001-9	0	520	4	0	16	0	256	0	0	52	0	8	0	0	0
15001-10	0	0	0	0	50	0	0	0	0	0	0	0	0	0	10
15001-11	0	180	32	0	30	0	56	0	0	0	0	0	0	0	0

[1]Received third dose of vaccine intragastrically.

[2]Received second, third, and fourth doses of vaccine intragastrically.

TABLE 8. ANTIBODY SECRETING CELL RESPONSES TO CS1 BY ELISPOT ASSAY AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28 (E. COLI CVD 15001)

Vaccinee	IgA					IgG					IgM				
	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35
15001-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-3[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-6[2]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-9	0	128	0	0	12	56	118	0	2	0	0	0	0	0	0
15001-10	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-11	0	140	0	0	0	0	34	0	0	0	2	0	0	0	0

[1]Received third dose of vaccine intragastrically.

[2]Received second, third, and fourth doses of vaccine intragastrically.

TABLE 9. ANTIBODY SECRETING CELL RESPONSES TO CS3 BY ELISPOT ASSAY AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28 (E. COLI CVD 15001)

Vaccinee	IgA					IgG					IgM				
	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35
15001-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-2	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0
15001-3[1]	0	0	0	0	26	0	0	0	0	0	0	0	0	0	0
15001-4	0	0	0	0	98	0	0	0	0	30	0	0	0	0	0
15001-6[1]	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-9	0	580	4	0	6	0	336	0	0	12	0	4	0	0	0
15001-10	0	0	0	0	88	0	0	0	0	0	0	0	0	0	0
15001-11	0	162	32	0	8	0	12	2	0	0	0	0	0	0	0

[1]Received third dose of vaccine intragastrically.

[2]Received second, third, and fourth doses of vaccine intragastrically.

TABLE 10. ANTIBODY SECRETING CELL RESPONSES TO CS3 PEPTIDE 795 BY ELISPOT ASSAY AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28 (E. COLI CVD 15001)

Vaccinee	IgA					IgG					IgM				
	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35
15001-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-3[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-6[2]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-11	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]Received third dose of vaccine intragastrically.

[2]Received second, third, and fourth doses of vaccine intragastrically.

TABLE 11. ANTIBODY SECRETING CELL RESPONSES TO CS3 PEPTIDE 792 BY ELISPOT ASSAY AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28 (E. COLI CVD 15001)

Vaccinee	IgA					IgG					IgM				
	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35	pre	+7	+14	+21	+35
15001-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-3[1]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-4	0	0	0	0	2	0	0	0	0	0	0	0	0	0	12
15001-6[2]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6
15001-8	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-9	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15001-11	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0

[1]Received third dose of vaccine intragastrically.

[2]Received second, third, and fourth doses of vaccine intragastrically.

TABLE 12. JEJUNAL FLUID SECRETORY IGA RESPONSES (RECIPROCAL TITER) TO CFA/II BY ELISA AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28 (E. COLI CVD 15001)

Vaccinee	Pre	+8	+14	+28	+35
15001-1	<4	<4	<4	<4	<4
15001-2	<4	IS	IS	IS	IS
15001-3[1]	4	<4	NS	4	<4
15001-4	4	<4	4	4	16[*]
15001-6[6]	4	NS	NS	NS	8
15001-7	<4	<4	4	4	32[*]
15001-8	8	4	4	8	8
15001-9	16	≥256	≥256	≥256	256[*]
15001-10	<4	<4	<4	<4	8[*]
15001-11	16	32	64	64	32[*]

[1]Received third dose of vaccine intragastrically.

[2]Received second, third, and fourth doses of vaccine intragastrically.

NS indicates no sample. IS indicates inadequate sample.

+ indicates significant rise in titer.

TABLE 13. SERUM ANTIBODY RESPONSES (RECIPROCAL TITERS) TO CFA/II BY ELISA AFTER VACCINATION WITH CFA/II VACCINE ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 21, AND 28 (E. COLI CVD 15001)

Vaccinee	IgG			IgA			IgM		
	Pre	+7	+28	Pre	+7	+28	Pre	+7	+28
15001-1	400	100	100	50	50	50	50	50	50
15001-2	6400	3200	6400	25	<25	<25	50	50	25
15001-3[1]	3200	6400	6400	100	200	50	100	50	50
15001-4	400	200	400	100	400	100*	100	50	50
15001-6[2]	1600	1600	1600	200	200	200	25	25	25
15001-7	400	6400	3200*	400	200	200	25	25	50
15001-8	3200	400	400	800	800	200	25	25	25
15001-9	6400	12800	6400	800	3200	3200*	50	50	50
15001-10	800	400	400	400	400	200	200	400	200
15001-11	800	1600	1600	400	400	100	25	25	25

[1]Received third dose of vaccine intragastrically.
[2]Received second, third, and fourth doses of vaccine intragastrically.
+ indicates significant rise in titer.

TABLE 14. SERUM ANTIBODY RESPONSES (RECIPROCAL TITERS) TO CS1 BY ELISA AFTER VACCINATION WITH CFA/II VACCINE ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 21, AND 28 (E. COLI CVD 15001)

Vaccinee	IgG			IgA			IgM		
	Pre	+7	+28	Pre	+7	+28	Pre	+7	+28
15001-1	<25	<25	<25	25	<25	<25	<25	<25	<25
15001-2	<25	<25	25	<25	<25	<25	<25	<25	<25
15001-3[1]	<25	<25	<25	<25	<25	<25	<25	<25	25
15001-4	<25	<25	<25	25	<25	<25	25	25	<25
15001-6[2]	<25	<25	<25	25	<25	<25	<25	<25	<25
15001-7	<25	<25	<25	<25	25	<25	<25	<25	<25
15001-8	<25	<25	<25	25	<25	25	<25	<25	<25
15001-9	800	800	400	200	200	200	<25	<25	<25
15001-10	<25	<25	<25	25	<25	<25	25	<25	25
15001-11	200	3200	800[+]	100	200	200	<25	<25	<25

[1]Received third dose of vaccine intragastrically.
[2]Received second, third, and fourth doses of vaccine intragastrically.
[+] indicates significant rise in titer.

TABLE 15. SERUM ANTIBODY RESPONSES (RECIPROCAL TITERS) TO CS3 BY ELISA AFTER VACCINATION WITH CFA/II VACCINE ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 21, AND 29 (E. COLI CVD 15001)

Vaccinee	IgG			IgA			IgM		
	Pre	+7	+28	Pre	+7	+28	Pre	+7	+28
15001-1	50	50	50	50	25	25	<25	25	<25
15001-2	800	1600	800	<25	<25	<25	25	25	25
15001-3[1]	<25	25	50*	50	25	25	25	50	50
15001-4	25	100	25*	50	50	50	<25	25	25
15001-6[2]	200	200	200	200	50	100	25	50	100*
15001-7	100	50	<25	100	50	25	50	<25	<25
15001-8	<25	200	100*	25	50	25	<25	50	50*
15001-9	100	800	800*	50	400	400*	25	50	25
15001-10	200	100	100	50	25	50	25	25	100*
15001-11	100	100	200	50	50	50	25	25	<25

[1]Received third dose of vaccine intragastrically.
[2]Received second, third, and fourth doses of vaccine intragastrically.
* Indicates significant rise in titer.

TABLE 16. CLINICAL AND BACTERIOLOGIC RESPONSES TO CHALLENGE WITH 5 x 10^7 CFU OF ENTEROTOXIGENIC E. COLI STRAIN E24377A (O139:H28 LT$^+$ST$^+$CS$^-$CS3$^+$) AMONG VACCINEES AND CONTROL VOLUNTEERS (E. COLI CVD 15002)

Volunteer	Incubation Period (hr:min)	Volume of Grade ≥ 3 Stools (ml)	No. of Stools Grade ≥ 3	Fever (Tmax) (°F)	Duration of Fecal Shedding (Days)	Peak 24hr Excretion (cfu/gm)
Vaccinees						
15001-1	19:20	1291	10	-	7	1×10^{9}
15001-2	46:30	837	3	-	6	1.7×10^{8}
15001-3	20:07	1231	9	+ (100.5)	5	3×10^{8}
15001-4	21:16	1052	7	+ (100.3)	7	1×10^{9}
15001-5	-	0	0	-	5	3×10^{6}
15001-7	18:16	4380	19	-	6	4×10^{8}
15001-8	25:19	9432	61	+ (101.9)	5	5×10^{8}
15001-9	-	0	0	-	5	1×10^{6}
15001-10	21:08	1608	14	-	7	3×10^{8}
15001-11	-	0	0	-	5	7.1×10^{7}
Mean	23:34	2819	14.7		5.8	3×10^{8}
Control Volunteers						
15002-1	19:54	1201	8	-	4	3×10^{8}
15002-5	24:01	872	6	-	8	3×10^{8}
15002-6	21:10	939	7	-	4	3×10^{8}
15002-8	12:58	1293	8	+ (101.4)	7	3×10^{9}
15002-9	22:12	1578	11	-	4	3×10^{8}
15002-11	27:04	1253	7	-	7	4×10^{8}
15002-12	20:31	2338	13	-	5	3×10^{9}
15002-13	21:58	740	5	-	8	5×10^{8}
15002-16	48:07	1004	7	-	6	1×10^{9}
15002-21	20:11	3468	18	-	5	5×10^{8}
Mean	23:38	1464	8.8		5.6	3.9×10^{8}

TABLE 17. ANTIBODY SECRETING CELL RESPONSES TO CFA/II, CS1, AND CS3 BY ELISPOT AFTER CHALLENGE WITH ENTEROTOXIGENIC E. COLI STRAIN E24377A AMONG VACCINEES AND CONTROL VOLUNTEERS (E. COLI CVD 15002)

Volunteer	CFA/II						CS1						CS3					
	IgA		IgG		IgM		IgA		IgG		IgM		IgA		IgG		IgM	
Vaccinees	Pre[3]	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7
15001 1	0	40	0	4	0	12	27	0	0	0	0	0	0	[illegible]	0	0	0	[illegible]
15001 2	0	0	630	7	0	0	0	0	0	0	0	0	0	[illegible]	0	0	0	0
15001 3[1]	12	202	40	34	0	20	0	[illegible]	0	0	0	0	0	[illegible]	0	72	0	56
15001 6	14	268	0	280	0	5	0	0	0	0	0	0	0	[illegible]	0	189	0	30
15001 8[2,4]	0	24	16	0	0	56	0	0	0	[illegible]	0	0	10	[illegible]	0	50	0	20
15001 9	0	12	0	36	40	20	0	0	0	0	0	0	0	[illegible]	0	[illegible]	0	0
15001 8	24	18	0	0	0	2	0	10	0	0	[illegible]	0	0	[illegible]	0	0	0	[illegible]
15001 9[4]	240	160	208	36	0	2	0	52	16	10	0	16	16	[illegible]	[illegible]	[illegible]	0	0
15001 10	36	348	0	232	0	88	0	0	0	0	0	0	0	[illegible]	0	140	0	10
15001 11[4]	0	36	0	2	0	2	12	16	0	24	0	0	0	[illegible]	0	0	0	0
Controls	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7
15002 1	0	200	610	621	0	20	0	100	0	152	0	0	0	[illegible]	0	640	0	30
15002 5	0	56	0	4	0	12	0	30	0	0	0	0	0	[illegible]	0	12	0	[illegible]
15002 6	0	140	0	36	0	144	0	0	0	0	0	0	0	[illegible]	0	0	0	[illegible]
15002 8	10	0	800	0	0	0	0	0	0	0	[illegible]	0	0	[illegible]	0	0	0	0
15002 9	32	208	0	208	0	14	0	240	0	168	0	0	[illegible]	[illegible]	0	148	0	22
15002 11	0	24	0	0	0	0	0	0	0	0	0	0	0	[illegible]	[illegible]	[illegible]	0	0
15002 12	0	408	0	312	0	240	0	0	0	0	0	0	0	[illegible]	0	480	0	102
15002 13	16	48	20	80	0	0	0	0	0	0	0	0	[illegible]	[illegible]	0	0	0	0
15002 16	0	32	0	280	0	20	0	0	0	0	0	0	0	[illegible]	0	200	0	[illegible]
15002 21	0	30	16	24	0	2	0	12	0	0	0	0	0	[illegible]	0	0	0	0

[1]Received third dose of vaccine intragastrically.
[2]Received second, third and fourth dose of vaccine orogastrically
[3]'Pre' indicates before challenge; for vaccinees, "pre" is day 57 after the first dose of vaccine
[4]Vaccinees who did not become ill

TABLE 18. ANTIBODY SECRETING CELL RESPONSES TO CS3 PEPTIDES 792 AND 795 BY ELISPOT AFTER CHALLENG WITH ENTEROTOXIGENIC E. COLI STRAIN E24377A AMONG VACCINEES AND CONTROL VOLUNTEERS (E. COLI CVD 15002)

Volunteer	CS3 PEPTIDE 792						CS3 PEPTIDE 795					
	IgA		IgG		IgM		IgA		IgG		IgM	
Vaccinees	Pre[3]	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7
15001-1	0	0	0	0	0	0	0	4	0	0	0	[illegible]
15001 2	0	0	0	0	0	0	4	0	0	0	0	[illegible]
15001 3[1]	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15001 4	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15001 6[2,4]	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15001-7	0	0	10	0	0	0	0	0	0	0	0	[illegible]
15001-8	22	2	0	0	0	0	0	0	0	0	0	[illegible]
15001-9[4]	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15001 10	2	0	0	0	0	0	2	0	0	0	0	[illegible]
15001-11[4]	0	10	0	0	0	0	0	12	0	0	0	[illegible]
Controls	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7
15002 4	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15002 5	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15002 6	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15002 8	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15002 9	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15002 11	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15002 12	0	0	0	0	0	0	2	0	0	0	0	[illegible]
15002 13	0	0	0	0	0	0	0	0	0	0	0	[illegible]
15002-16	2	10	0	0	0	0	2	0	0	0	0	[illegible]
15002 21	0	0	0	0	0	0	0	0	0	0	0	[illegible]

[1]Received third dose of vaccine intragastrically
[2]Received second, third, and fourth doses of vaccine intragastrically
[3]'Pre' indicates before challenge; for vaccinees, 'pre' is day 57 after first dose of vaccine
[4]Vaccinees who did not become ill

TABLE 19. ANTIBODY SECRETING CELL RESPONSES TO 0139 LIPOPOLYSACCHARIDE (LPS) AND HEAT LABILE ENTEROTOXIN (LT) BY ELISPOT AFTER CHALLENGE WITH ENTEROTOXIGENIC E. COLI STRAIN E24377A AMONG VACCINEES AND CONTROL VOLUNTEERS (E. COLI CVD 15002)

Volunteer	O139 LPS						LT					
	IgA		IgG		IgM		IgA		IgG		IgM	
Vaccinees	Pre[3]	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7
15001-1	19	176	0	112	0	48	22	84	0	1	0	0
15001-2	0	0	0	4	0	48	0	0	0	0	0	0
15001-3[1]	0	200	0	0	0	16	0	0	0	0	0	0
15001-4	0	520	0	480	0	320	0	88	0	160	0	0
15001-6[2,4]	0	198	0	0	0	96	0	88	0	152	2	4
15001-7	0	324	0	4	0	0	0	44	0	208	0	0
15001-8	24	240	0	0	0	24	0	4	0	0	0	0
15001-9[4]	0	28	0	56	0	0	0	4	0	0	0	0
15001-10	2	200	0	0	0	0	0	4	0	24	0	0
15001-11[4]	0	80	0	0	0	0	0	40	0	0	0	2
Controls	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7	Pre	+7
15002-5	0	168	0	208	0	42	0	0	0	0	0	0
15002-5	0	400	0	120	0	288	0	224	0	512	0	0
15002-6	0	432	0	36	0	144	0	2	0	0	0	0
15002-8	0	327	0	144	0	160	0	800	0	640	0	400
15002-9	0	168	0	0	0	120	0	12	0	24	0	0
15002-11	0	440	0	120	0	288	0	6	0	512	0	0
15002-12	0	432	0	0	0	48	0	24	0	32	0	0
15002-13	0	320	0	0	0	104	0	240	0	160	0	0
15002-18	0	224	0	80	0	48	0	216	0	208	1	0
15002-21	0	184	0	0	0	16	0	0	0	0	0	0

[1]Received third dose of vaccine homogenously
[2]Received second, third, and fourth doses of vaccine homogenously
[3]'Pre' indicates before challenge; for vaccinees, 'pre' is day 57 after last dose of vaccine
[4]Vaccinees who did not become ill

TABLE 20. IMMUNE RESPONSES AS MEASURED BY ANTIBODY SECRETING CELLS (ASC) AND BY JEJUNAL FLUID SECRETORY IGA AFTER VACCINATION WITH CFA/II ENCAPSULATED IN BIODEGRADABLE MICROSPHERES ON DAYS 0, 7, 14, AND 28

Immunologic Assay	Number of Responders[1]	Geometric mean peak number of spots per 10^6 PBMC (ASC) or reciprocal antibody titer (sIgA)
ASC IgA anti-CFA/II	5/10	44
ASC IgA anti-CS1	3/10	48
ASC IgA anti-CS3	5/10	116
Jejunal fluid sIgA anti-CFA/II	5/10	42

[1]Responses that had occurred by day 35 after the first dose of vaccine, i.e., day 7 after the fourth dose

TABLE 21. IMMUNE RESPONSES AFTER WILD-TYPE ETEC CHALLENGE AS MEASURED BY ANTIBODY SECRETING CELLS (ASC) AND BY JEJUNAL FLUID SECRETORY IGA IN UNIMMUNIZED CONTROL VOLUNTEERS

Immunologic Assay	Number of Responders[1]	Geometric mean peak number of spots per 10^6 PBMC (ASC) or reciprocal antibody titer (sIgA)
ASC IgA anti-CFA/II	9/10	88
ASC IgA anti-CS1	4/10	58
ASC IgA anti-CS3	9/10	161
Jejunal fluid sIgA anti-CFA/II	6/9	72

[1]Measured day 7 after challenge

TABLE 22. PRE-CHALLENGE IMMUNITY AND CLINICAL AND BACTERIOLOGIC RESPONSE TO CHALLENGE WITH 5 X 10^9 CFU OF ENTEROTOXIGENIC E. COLI STRAIN E24377A (0139:H28 $LT^+ST^+CS1^+CS3^+$) AMONG VACCINEES AND CONTROL VOLUNTEERS

	Vaccinees	Controls
Number with >4 IgA anti-colonization factor ASC[1] per 10^6 PBMC on the day of challenge[2]	8/10	4/10
Geometric mean number of IgA anti-colonization factor ASC per 10^6 PBMC on the day of challenge[3]	25	14
Attack Rate for Diarrhea	7/10	10/10
Volume of Diarrhea	2819 ml	1464 ml
Peak Stool Excretion of Challenge Organism	3 x 10^8 cfu	4 x 10^8 cfu

[1]Including anti-CFA/II, anti-CS1, and/or anti-CS3

[2]Day 57 after the first dose of vaccine

[3]Among those with >4 IgA ASC before challenge (n=8 for vaccinees and n=4 for controls)

DISCUSSION

The potential advantage of microcapsules lies in their ability to be programmed during fabrication into forms that have quite difference release profiles, including slow and steady release, multiple bursts of antigen over a period of time, or combinations of release forms. Sieving allows choice of microcapsule size, and the ability of DL-PLG to sequester antigen from the host's immune system until release occurs enhances control over exposure of the recipient's immune system to antigen over a sustained period of time. These characteristics provided the impetus for these studies as they indicate potential for achieving the effects of a multiple injection regimen by controlling release in vivo after a single injection.

The results of these studies are important for gaining an under standing of the fundamental differences between the manner in which alum and microcapsules interact with the immune system. The antigen release studies showed that alum firmly bound the antigen on its surface, whereas the microcapsules sequestered the antigen load within the interstices of an immunologically inert polymer. Release of antigen from microcapsules was spontaneous and gradual while antigen release from alum wa probably enzymatically mediated within host macrophages. Alum thus performed at least two useful functions as an adjuvant: by bearing its entire load of antigen upon its surface, it provided a large single exposure of antigen to the host; and, by being

readily phagocytized by host macrophages, it served as a means of targeting the antigen to the immune system.

In order for microcapsules to be efficacious as a vaccine delivery system, a means of incorporating the two properties common to alum adjuvant must be devised. These properties, which where discussed above, are targeting antigen to the immune system and delivering the antigen load in a single concentrated pulse at its target. A gradual, sustained release of free antigen, as was achieved with the 100 micron microcapsules used in these studies, could be expected to elicit an immune response similar to that seen with either regimen b or regimen c (Table 5), where multiple injections of small doses were employed. In fact, as shown in Table 3, the microencapsulated immunogen elicited a response similar to that achieved with regimen b. This is probably due to the fact that the microcapsules release approximately 10% of their antigenic load immediately after injection.

Microcapsules with extended release patterns tend to be large (>10 microns in diameter) and thus fail to be readily phagocytized. In order for the larger microcapsules with prolonged antigen release characteristics to be efficacious, the antigen eventually released from those microcapsules would have be in a form which targeted and concentrated it within the recipient's immune system. This might be effectively achieved by microencapsulation of antigen coated alum or by

microencapsulating clusters of smaller (<10 microns) microcapsules.

Microcapsules under 10 microns in diameter tend to be readily phagocytized and also tend to under go rapid spontaneous degradation due to their high surface to volume ratio. These smaller microcapsules would be well suited for eliciting a primary response if their pulse of antigen release could be programmed to occur after phagocytosis.

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We Claim:

1. An immunostimulating composition comprising encapsulating- microspheres, which may contain a pharmaceutically-acceptable adjuvant, wherein said microspheres having a diameter between 1 nanogram (ng) to 10 microns (um) are comprised of (a) a biodegradable-biocompatible poly (DL-lactide-co-glycolide) as the bulk matrix, wherein the relative ratio between the amount of lactide and glycolide components are within the range of 40:60 to 0:100 and (b) an immunogenic substance comprising Colony Factor Antigen (CFA/II), hepatitis B surface antigen (HBsAg), or a physiologically similar antigen that serves to elicit the production of antibodies in animal subjects.

2. An immunostimulating composition according to Claim 1 wherein the amount of said immunogenic substance is within the range of 0.1 to 1.5% based on the volume of said bulk matrix.

3. An immunostimulating composition according to Claim 2 wherein the relative ratio between the lactide and glycolide component is within the range of 48:52 to 58:42.

4. An immunostimulating composition according to Claim 2 wherein the size of more than 50% of said microspheres is between 5 to 10 um in diameter by volume.

5. An immunostimulatiang composition according to Claim 1 wherein the immunogenic substance is the synthetic peptide representing the peptide fragment beginning with the amino acid residue 63 through 78 of Pilus Protein CS3, said residue having the amino acid sequence, 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-His-Glu-Thr-Asn-Asn-Ser-Ala).

6. A vaccine comprising an immunostimulating composition of Claim 4 and a sterile, pharmaceutically-acceptable carrier therefor.

7. A vaccine comprising an immunostimulating composition of Claim 6 wherein said immunogenic substance is Colony Factor Antigen (CFA/II).

8. A vaccine comprising an immunostimulating composition of Claim 6 wherein said immunogenic substance is hepatitis B surface antigen (HBsAg).

9. A method for the vaccination against bacterial infection comprising administering to a human, an antibactericidally effective amount of a composition of Claim 6.

10. A method according to Claim 8 wherein the bacterial infection is caused by a bacteria selected from the group consisting essentially of Salmonella typhi, Shigella Sonnei,

Shigella Flexneri, Shigella dysenteriae, Shigella boydii, Escheria coli, Vibrio cholera, yersinia, staphylococus, clostridium, and campylobacter.

11. A method for the vaccination against viral infection comprising administering to a human an antivirally effective amount of a composition of Claim 8.

12. A diagnostic assay for bacterial infections comprising a composition of Claim 4.

13. A method of preparing an immunotherapeutic agent against infections caused by a bacteria comprising the step of immunizing a plasma donor with a vaccine according to Claim 7 such that a hyperimmune globulin is produced which contains antibodies directed against the bacteria.

14. A method preparing an immunotherapeutic agent against infections caused by a virus comprising the step of immunizing a plasma donor with a vaccine according to Claim 8 such that hyperimmune globulin is produced which contains antibodies directed against the hepatitis B virus.

15. An immunotherapy method comprising the step of administering to a subject an immunostimulatory amount of hyperimmune globulin prepared according to Claim 13.

16. An immunotherapy method comprising the step of administering to a subject an immunostimulatory amount of hyperimmune globulin prepared according to Claim 14.

17. A method for the protection against infection of a subject by enteropathogenic organisms or hepatitis B virus comprising administering to said subject an immunogenic amount of an immunostimulating composition of Claim 3.

18. A method according to Claim 17 wherein the immunostimulating composition is administered orally.

19. A method according to Claim 17 wherein the immunostimulating composition is administered parenterally.

20. A method according to Claim 17, wherein the immunostimulating composition is administered in four separate doses on day 0, day 7, day 14, and day 28.

21. A method according to Claim 17 wherein the immunogenic substance is the synthetic peptide representing the peptide fragment beginning with the amino acid residue 63 through 78 of Pilus Protein CS3 said residue having the amino acid sequence 63(Ser-Lys-Asn-Gly-Thr-Val-Thr-Try-Ala-His-Glu-Thr-Asn-Asn-Ser-Ala).

Figure 1

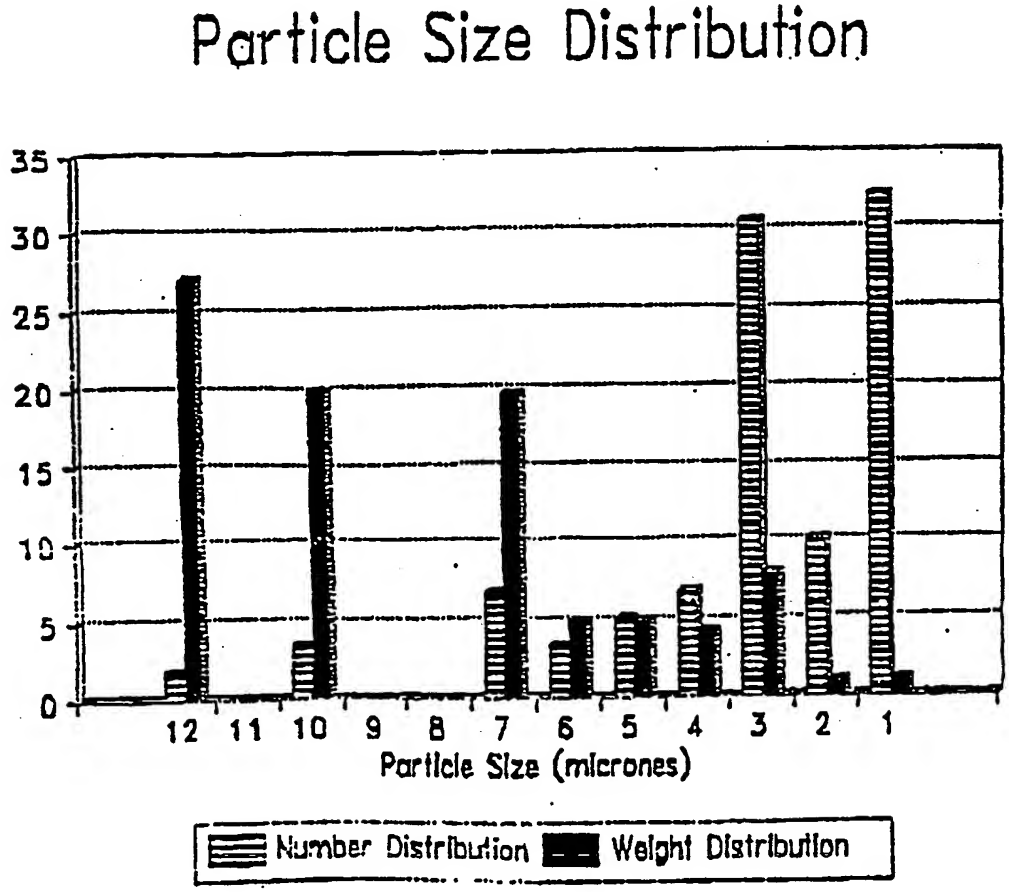

Particle Size Distribution
35
30
25
20
15
10
5
0
12 11 10 9 8 7 6 5 4 3 2 1
Particle Size (microns)
Number Distribution
Weight Distribution

FIGURE 2

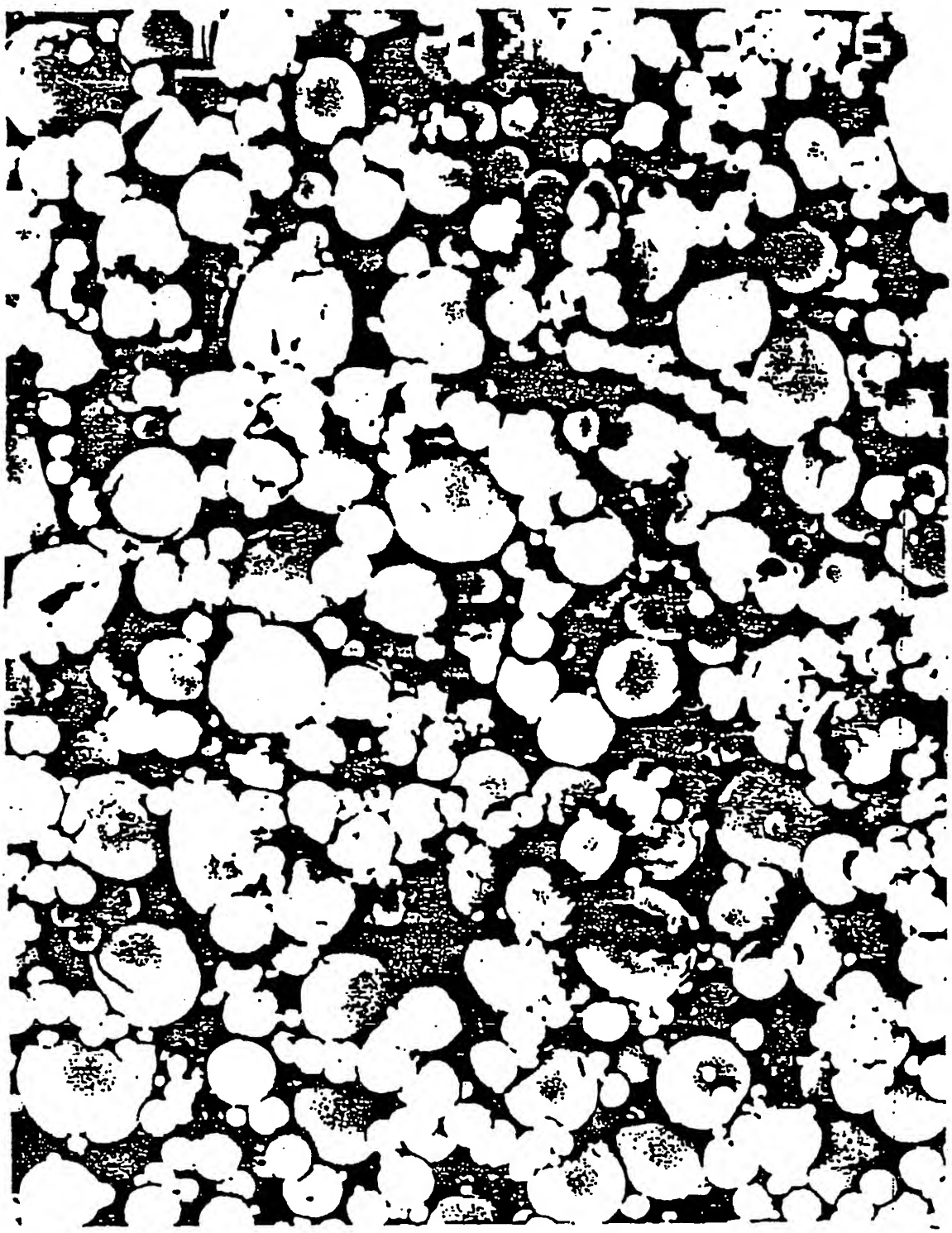


Fig. 3(a)

Spleen

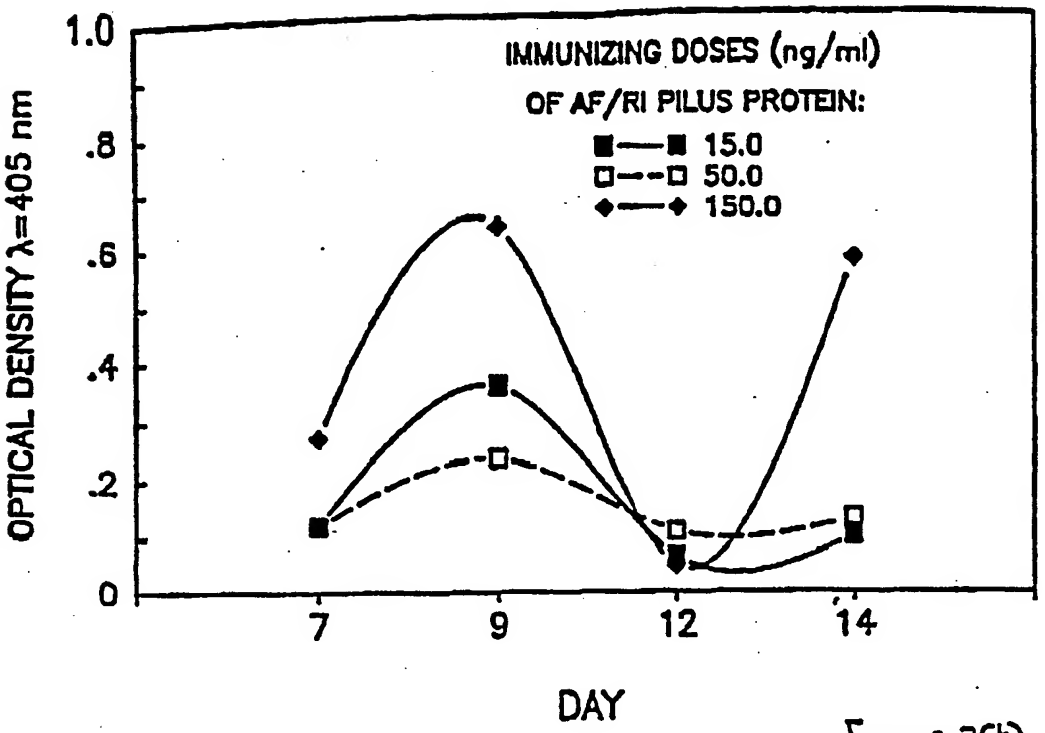


Figure 3(b)

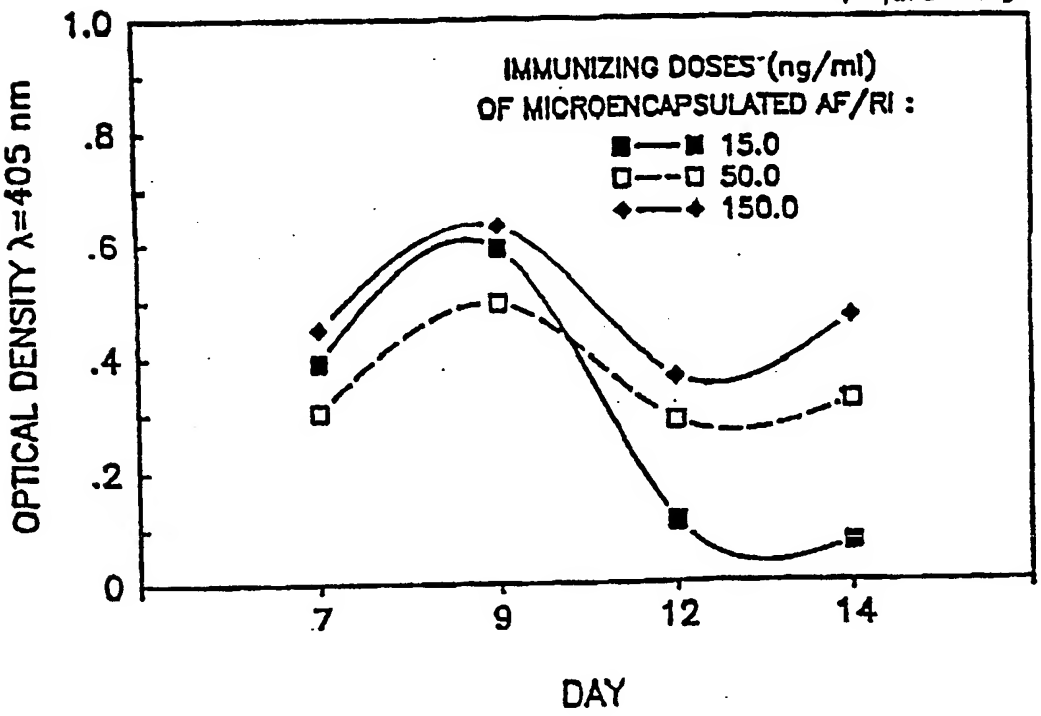


Figure 4(a)

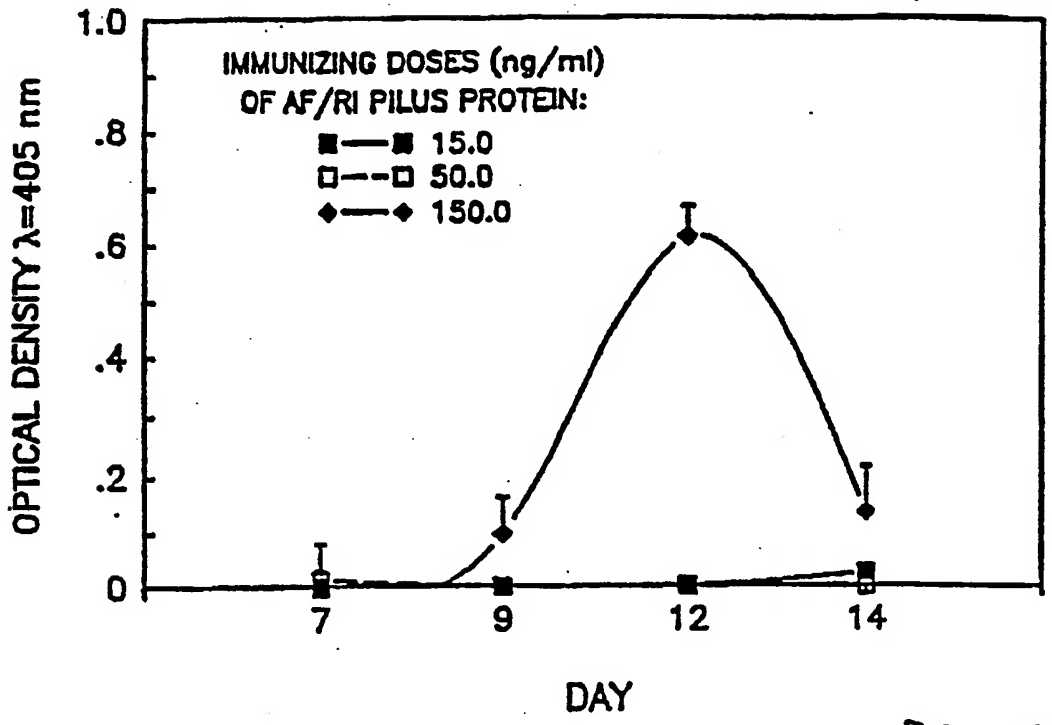
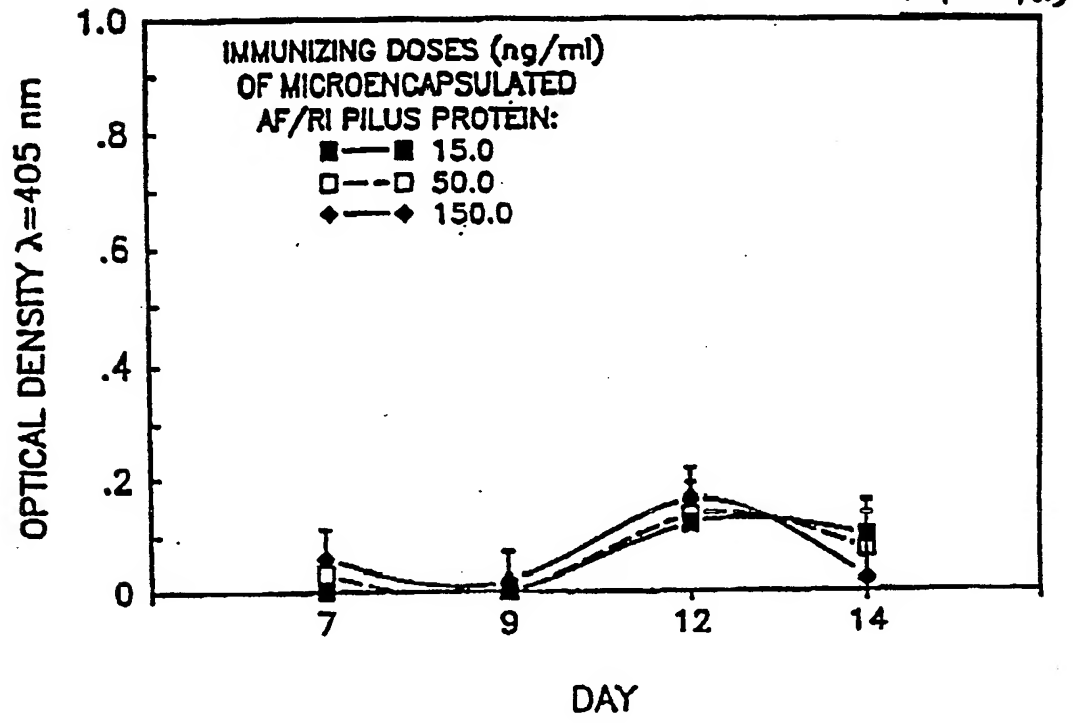


Figure 4(b)



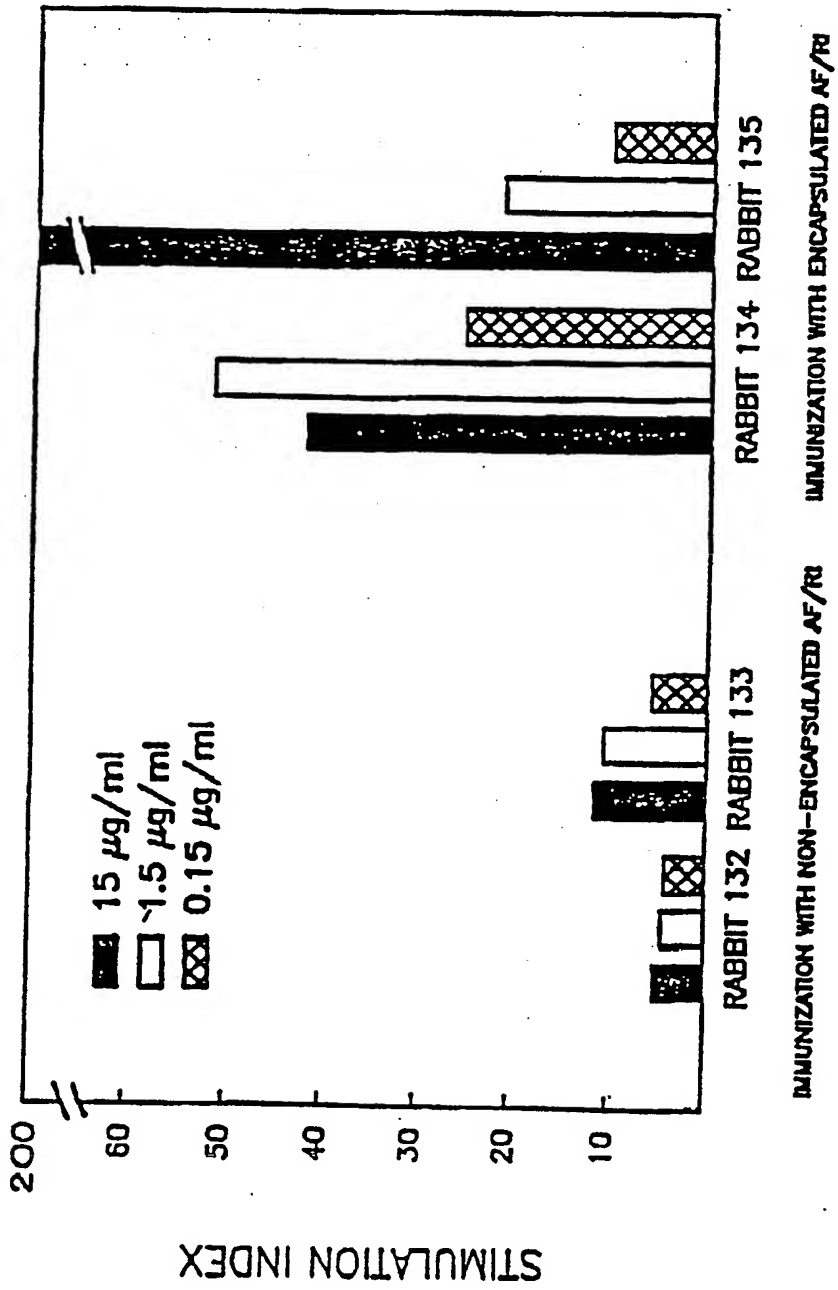

STIMULATION INDEX
200
60
50
40
30
20
10
15 µg/ml
1.5 µg/ml
0.15 µg/ml
RABBIT 132
RABBIT 133
RABBIT 134
RABBIT 135
IMMUNIZATION WITH NON-ENCAPSULATED AF/R2
IMMUNIZATION WITH ENCAPSULATED AF/R2

Figure 5

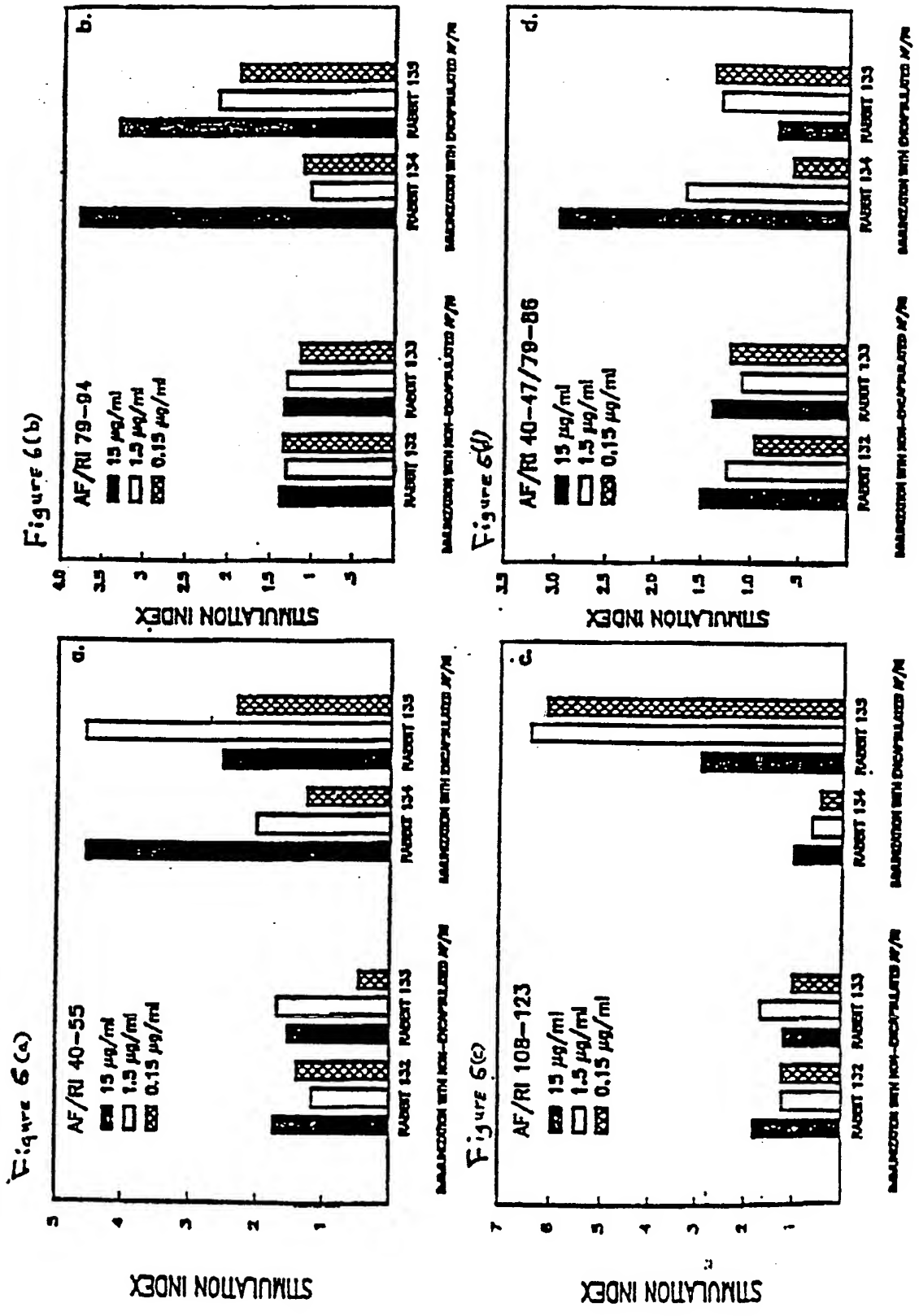


Figure 6(a)
AF/RI 40-55
15 µg/ml
1.5 µg/ml
0.15 µg/ml
STIMULATION INDEX
RABBIT 132
RABBIT 133
RABBIT 134
RABBIT 135
Figure 6(b)
AF/RI 79-94
15 µg/ml
1.5 µg/ml
0.15 µg/ml
STIMULATION INDEX
Figure 6(c)
AF/RI 108-123
15 µg/ml
1.5 µg/ml
0.15 µg/ml
STIMULATION INDEX
Figure 6(d)
AF/RI 40-47/79-86
15 µg/ml
1.5 µg/ml
0.15 µg/ml
STIMULATION INDEX

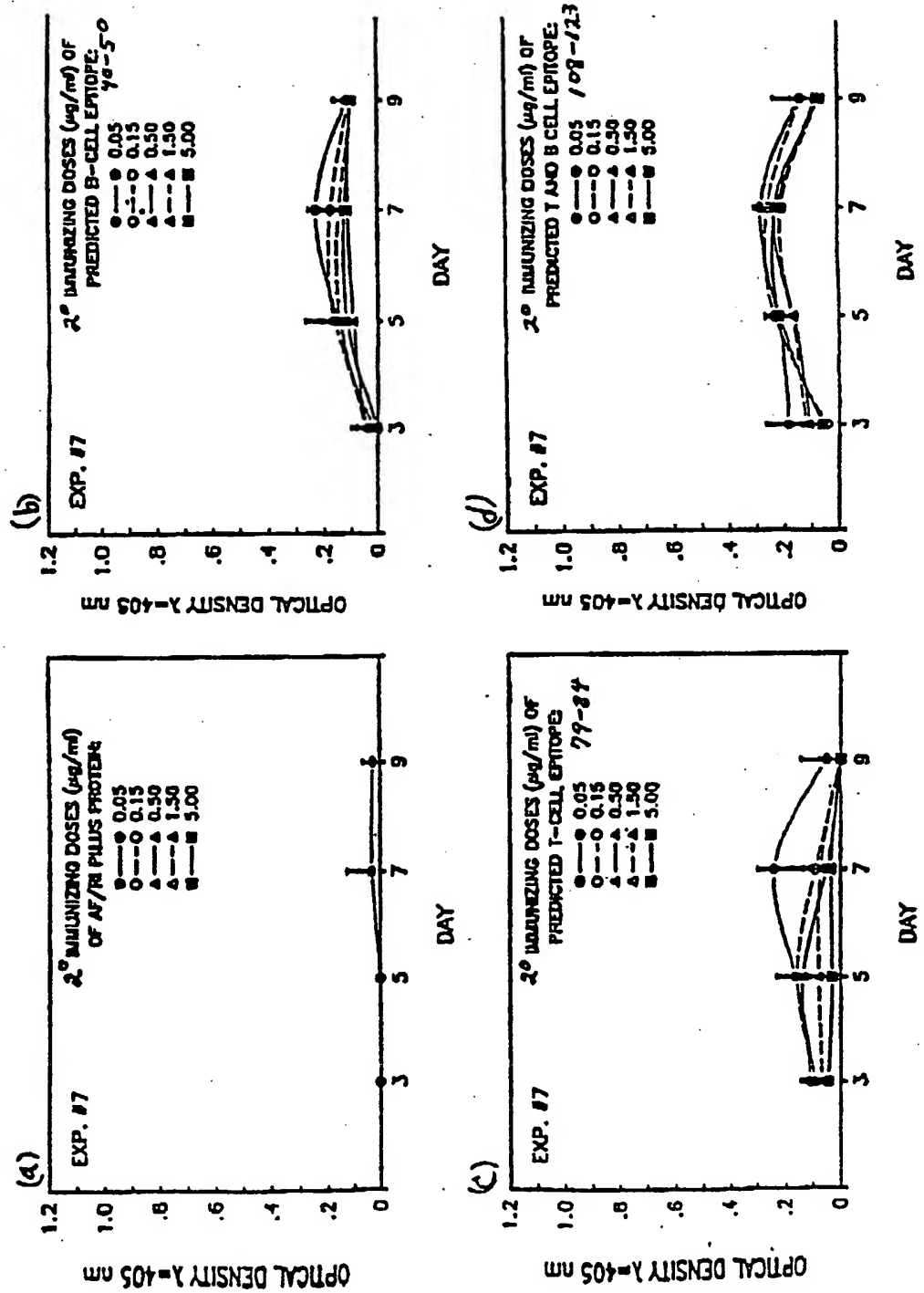


Fig. 7
Peyer's patch

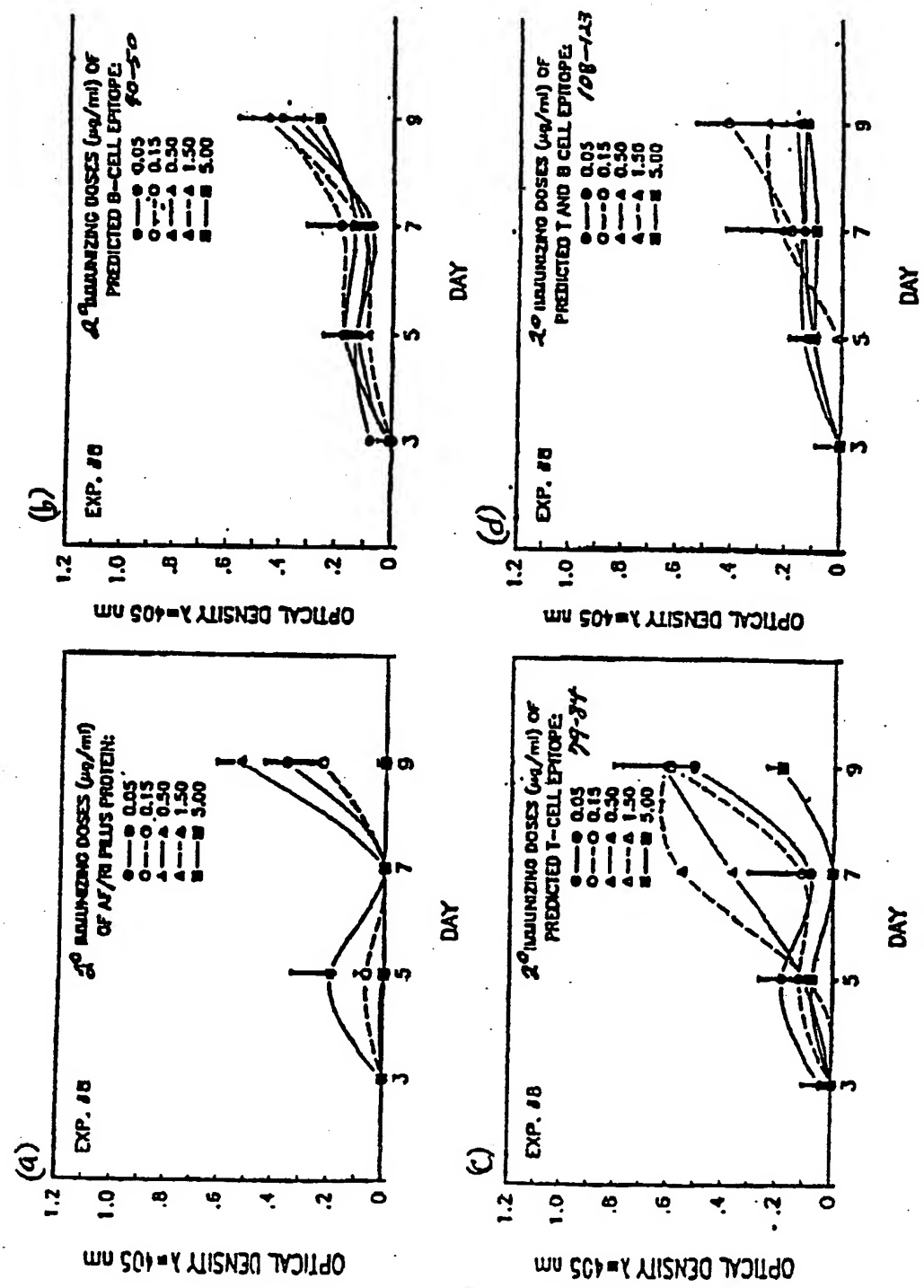

(b)
EXP. #8
2° IMMUNIZING DOSES (μg/ml) OF PREDICTED B-CELL EPITOPE: 50-59
0.05
0.15
0.50
1.50
5.00
OPTICAL DENSITY λ=405 nm
DAY
(d)
EXP. #8
2° IMMUNIZING DOSES (μg/ml) OF PREDICTED T AND B CELL EPITOPE: 108-123
0.05
0.15
0.50
1.50
5.00
OPTICAL DENSITY λ=405 nm
DAY
(a)
EXP. #8
2° IMMUNIZING DOSES (μg/ml) OF AF/R8 PLUS PROTEIN:
0.05
0.15
0.50
1.50
3.00
OPTICAL DENSITY λ=405 nm
DAY
(c)
EXP. #8
2° IMMUNIZING DOSES (μg/ml) OF PREDICTED T-CELL EPITOPE: 29-39
0.05
0.15
0.50
1.50
5.00
OPTICAL DENSITY λ=405 nm
DAY

Fig. 8

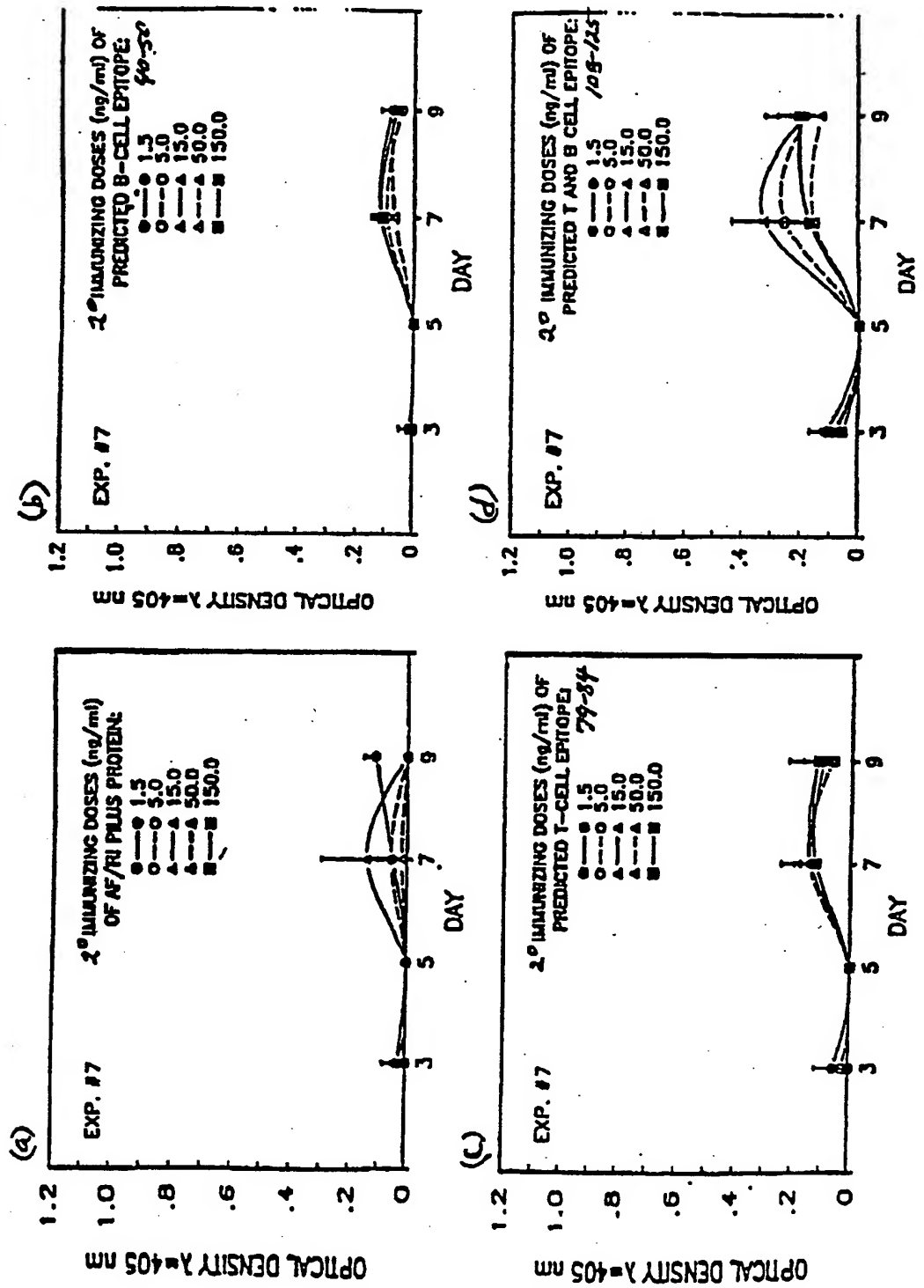

(a)
EXP. #7
2° IMMUNIZING DOSES (ng/ml) OF AF/RI PLUS PROTEIN:
1.5
5.0
15.0
50.0
150.0
OPTICAL DENSITY λ=405 nm
DAY
(b)
EXP. #7
2° IMMUNIZING DOSES (ng/ml) OF PREDICTED B-CELL EPITOPE: 40-50
1.5
5.0
15.0
50.0
150.0
OPTICAL DENSITY λ=405 nm
DAY
(c)
EXP. #7
2° IMMUNIZING DOSES (ng/ml) OF PREDICTED T-CELL EPITOPE: 79-84
1.5
5.0
15.0
50.0
150.0
OPTICAL DENSITY λ=405 nm
DAY
(d)
EXP. #7
2° IMMUNIZING DOSES (ng/ml) OF PREDICTED T AND B CELL EPITOPE: 105-115
1.5
5.0
15.0
50.0
150.0
OPTICAL DENSITY λ=405 nm
DAY

Fig. 9

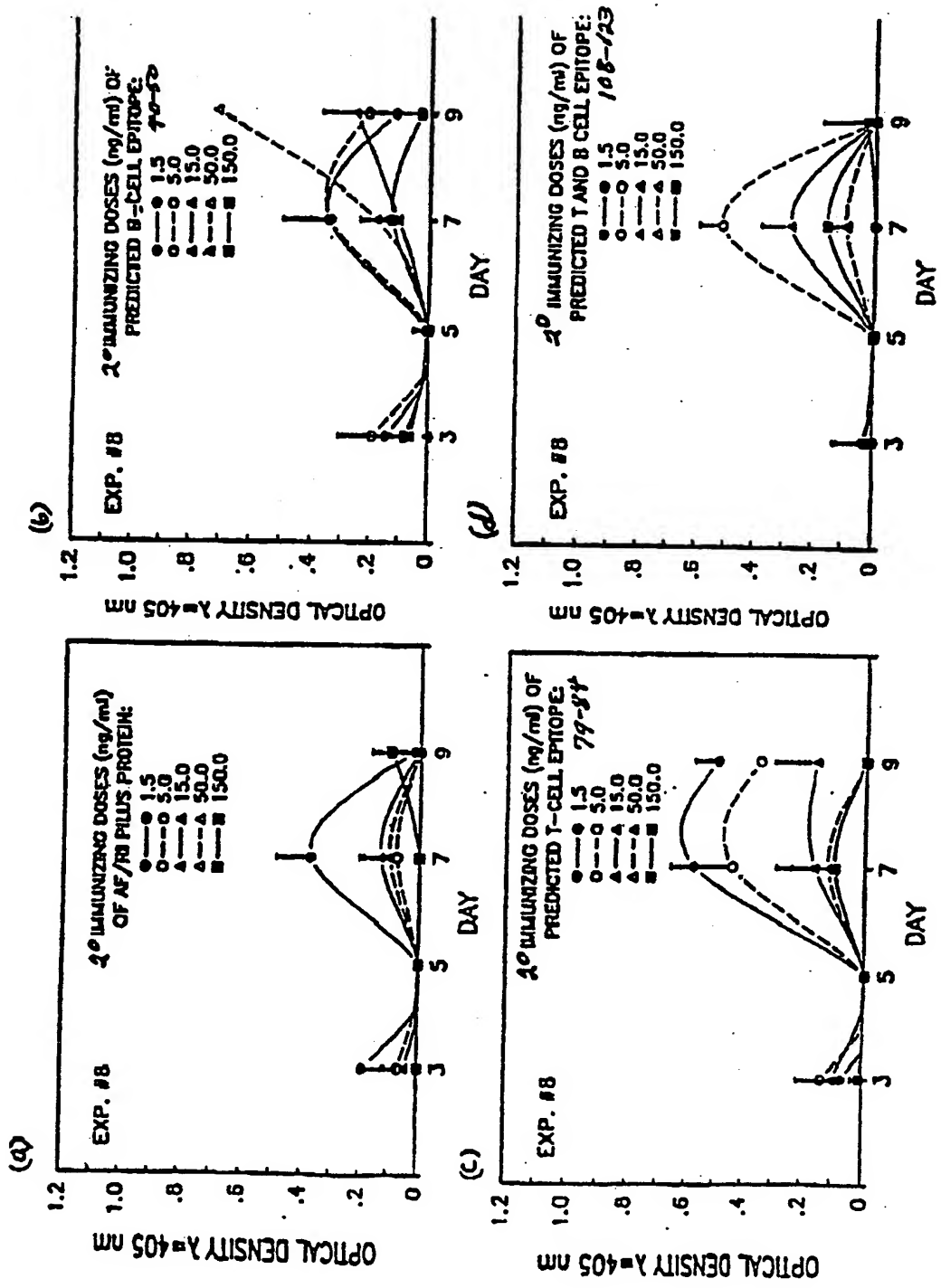

(a)
EXP. #8
2° IMMUNIZING DOSES (ng/ml) OF AF/RB PILUS PROTEIN:
1.5
5.0
15.0
50.0
150.0
OPTICAL DENSITY λ=405 nm
DAY
(b)
EXP. #8
2° IMMUNIZING DOSES (ng/ml) OF PREDICTED B-CELL EPITOPE: 70-80
(c)
EXP. #8
2° IMMUNIZING DOSES (ng/ml) OF PREDICTED T-CELL EPITOPE: 79-91
(d)
EXP. #8
2° IMMUNIZING DOSES (ng/ml) OF PREDICTED T AND B CELL EPITOPE: 108-123

Fig. 10

Fig. 1

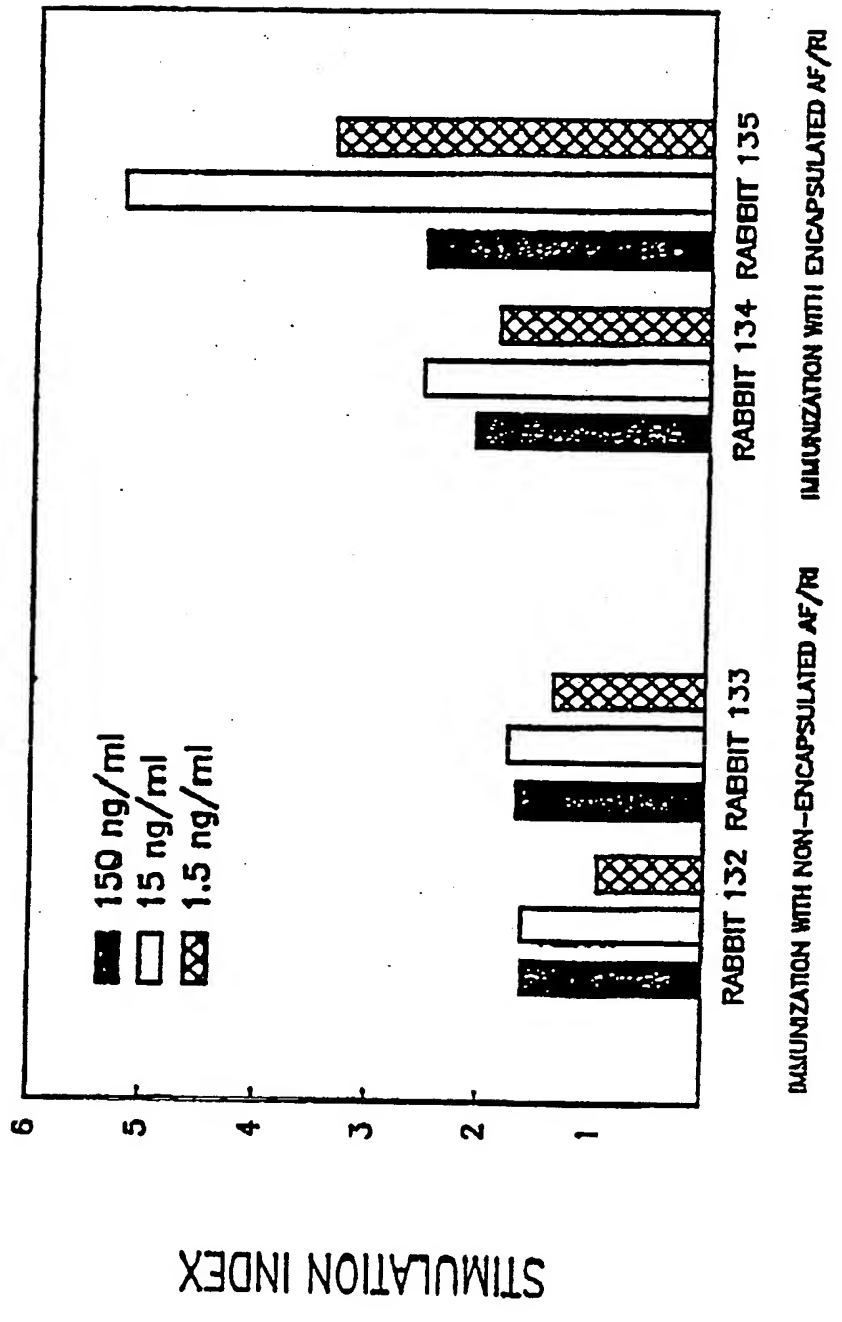

STIMULATION INDEX
6
5
4
3
2
1
150 ng/ml
15 ng/ml
1.5 ng/ml
RABBIT 132
RABBIT 133
IMMUNIZATION WITH NON-ENCAPSULATED AF/RI
RABBIT 134
RABBIT 135
IMMUNIZATION WITH ENCAPSULATED AF/RI

Fig. 12

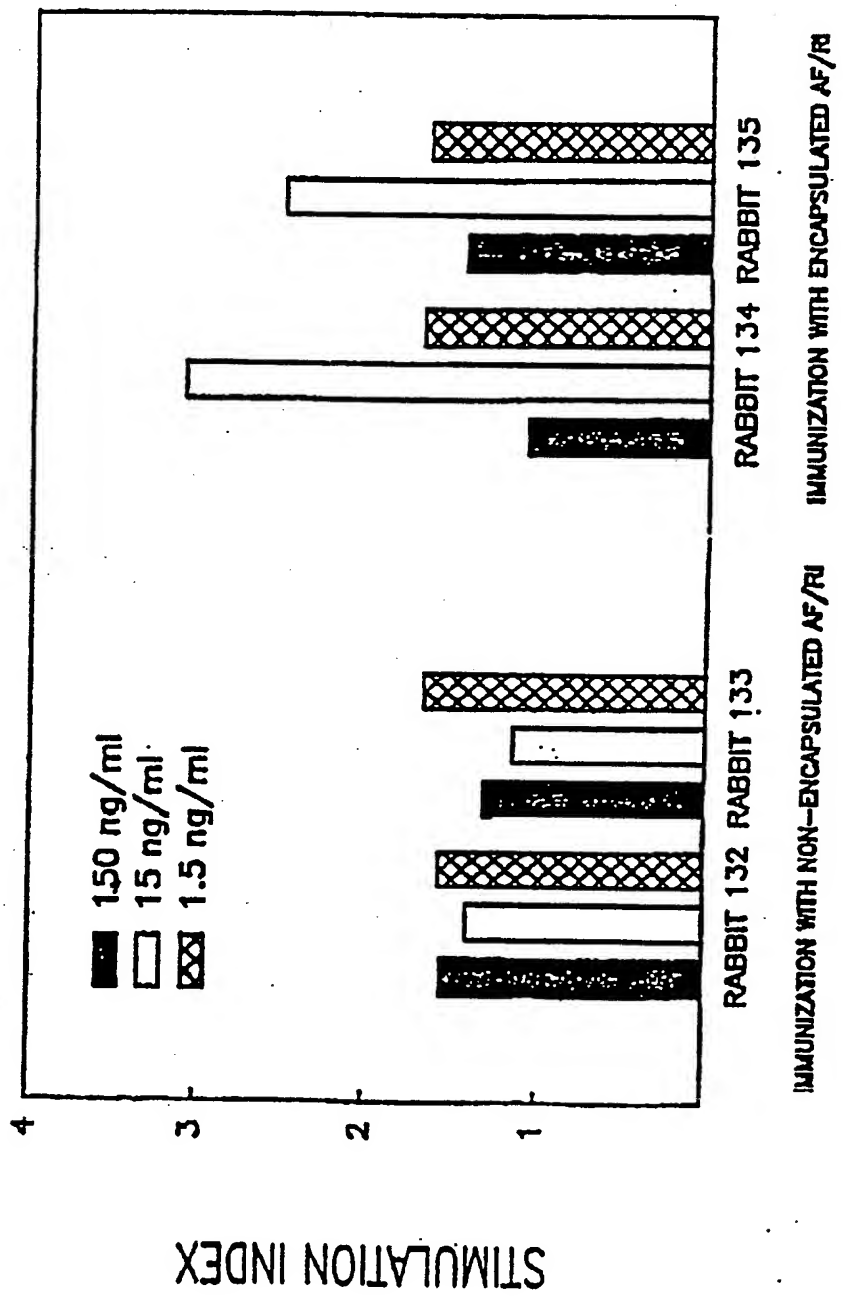

STIMULATION INDEX
4
3
2
1
150 ng/ml
15 ng/ml
1.5 ng/ml
RABBIT 132
RABBIT 133
RABBIT 134
RABBIT 135
IMMUNIZATION WITH NON-ENCAPSULATED AF/RI
IMMUNIZATION WITH ENCAPSULATED AF/RI

Fig 13

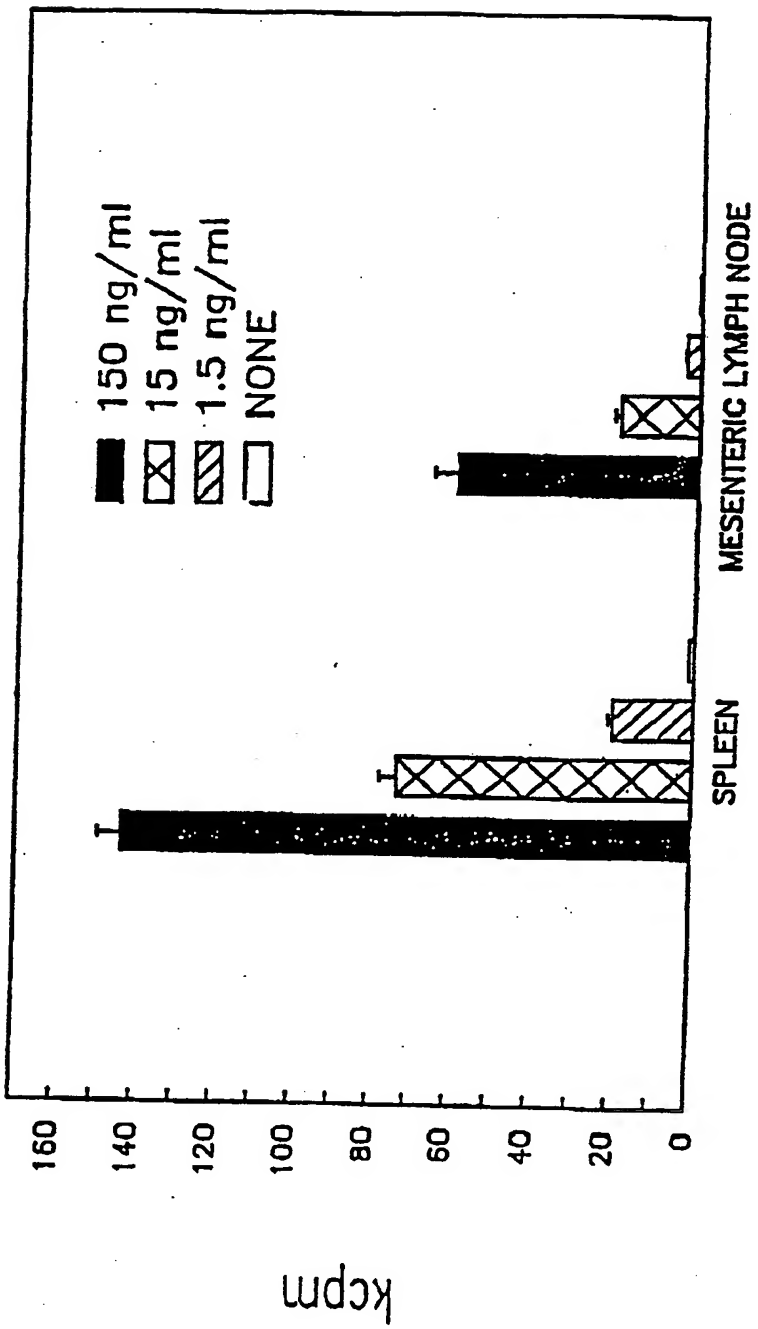

150 ng/ml
15 ng/ml
1.5 ng/ml
NONE
MESENTERIC LYMPH NODE
SPLEEN
kcpm
160
140
120
100
80
60
40
20
0

Fig. 14

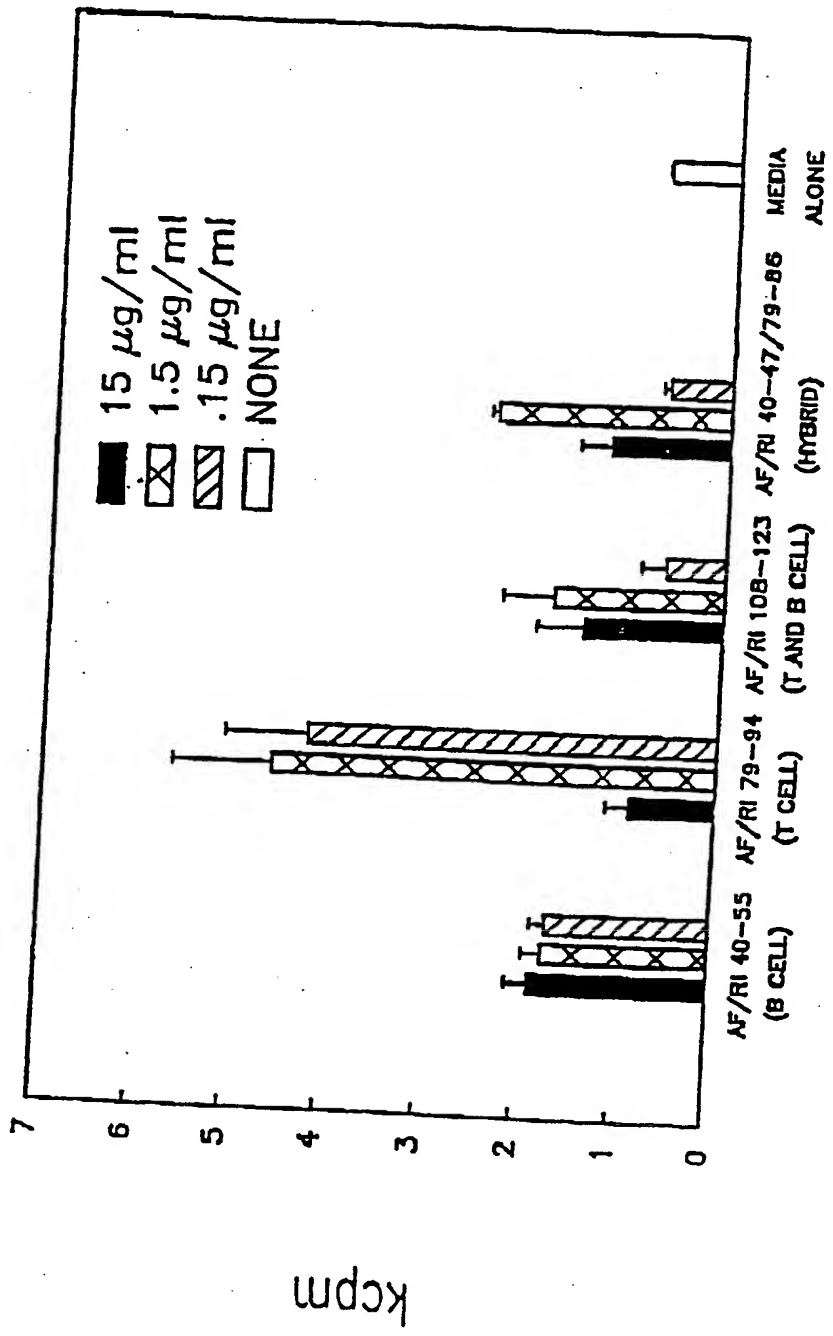
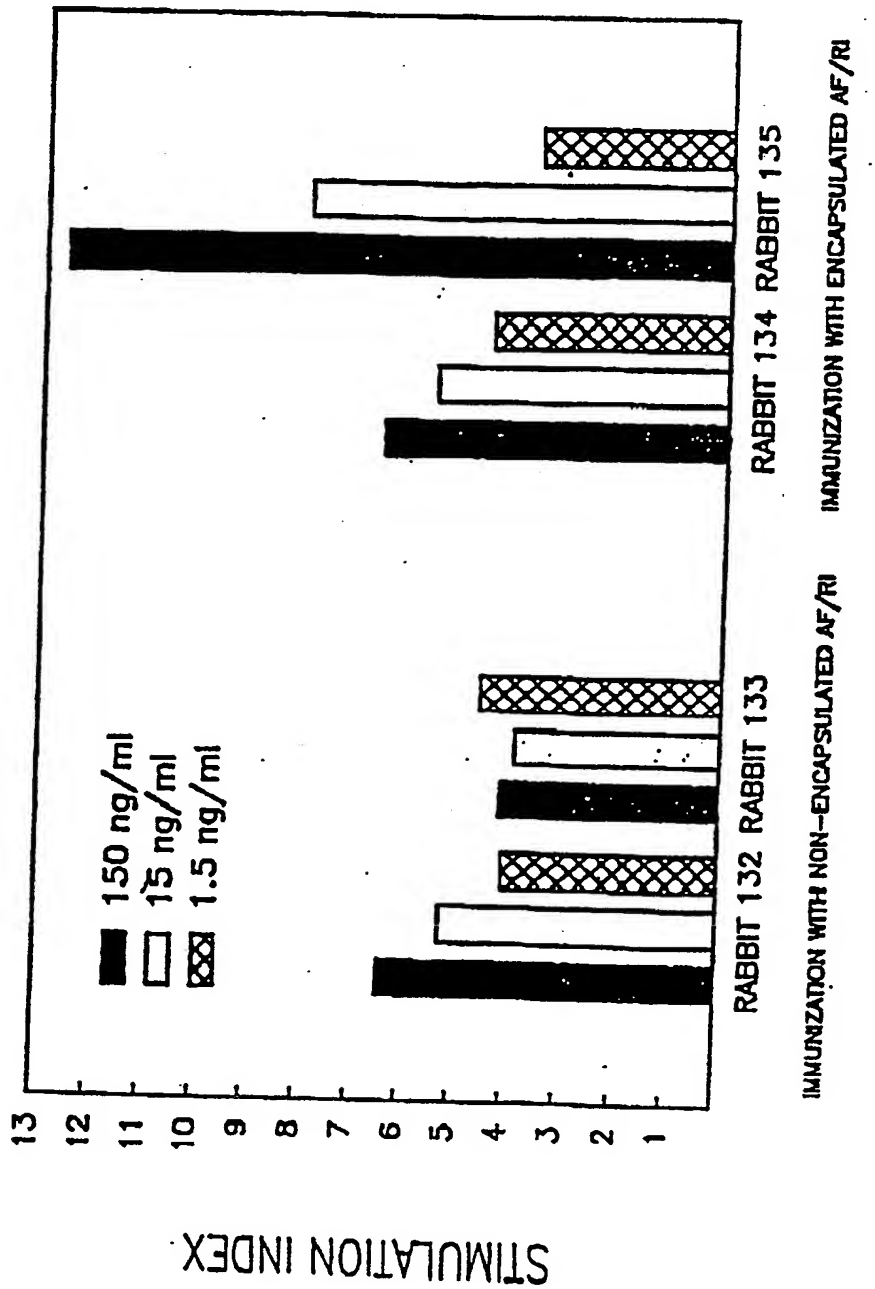
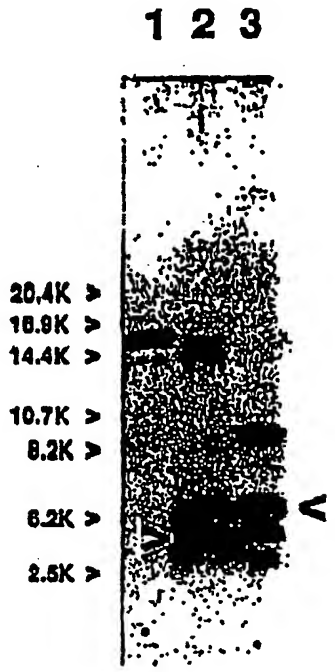

15 μg/ml
1.5 μg/ml
.15 μg/ml
NONE
kcpm
0
1
2
3
4
5
6
7
AF/RI 40-55 (B CELL)
AF/RI 79-94 (T CELL)
AF/RI 108-123 (T AND B CELL)
AF/RI 40-47/79-86 (HYBRID)
MEDIA ALONE

Fig. 15


STIMULATION INDEX
13
12
11
10
9
8
7
6
5
4
3
2
1
150 ng/ml
15 ng/ml
1.5 ng/ml
RABBIT 132
RABBIT 133
RABBIT 134
RABBIT 135
IMMUNIZATION WITH NON-ENCAPSULATED AF/RI
IMMUNIZATION WITH ENCAPSULATED AF/RI

A



B

Lane 2	LADTPQLTDVLNSTVQMP	(62-79)
Lane 3	SYRVMTQVHTNQATKKVIV	(42-60)

Figure 16

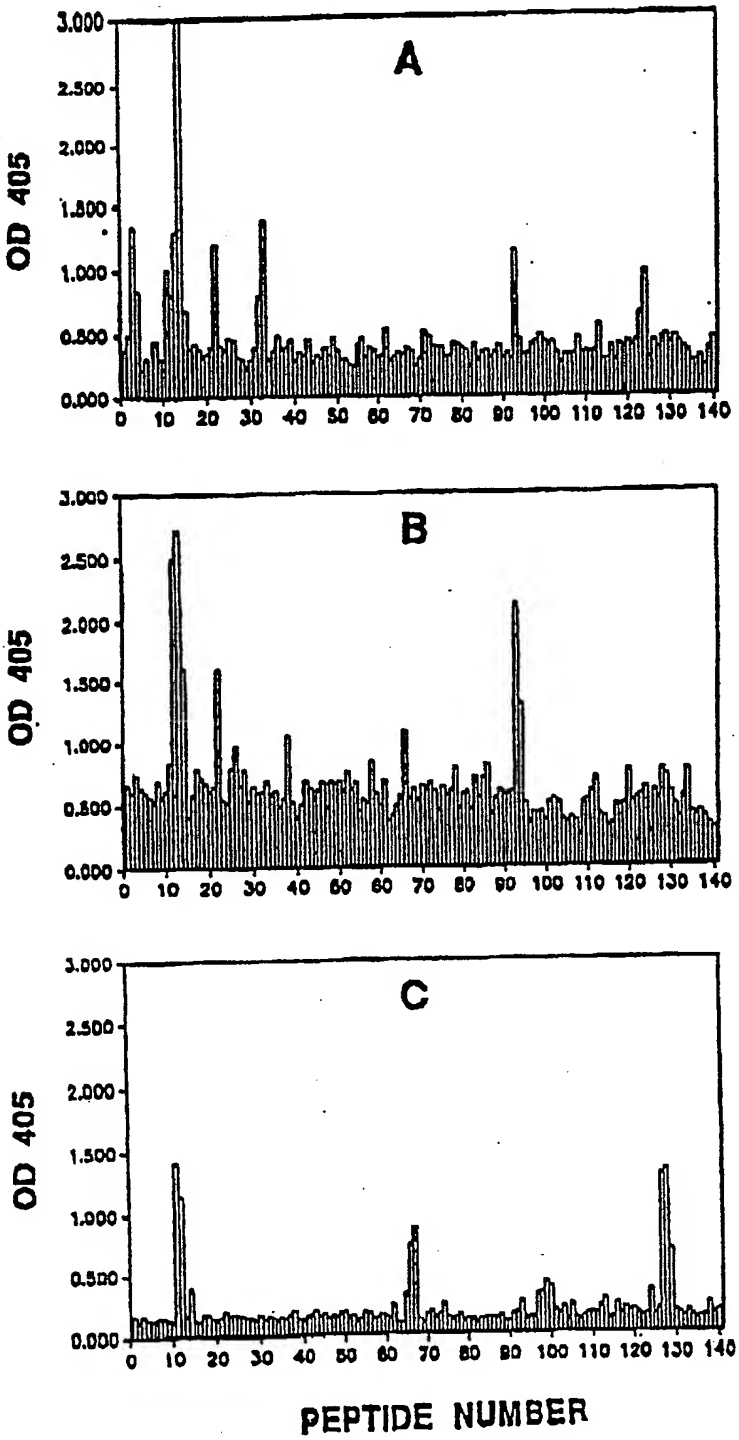

A
B
C
OD 405
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140
PEPTIDE NUMBER

Figure 19

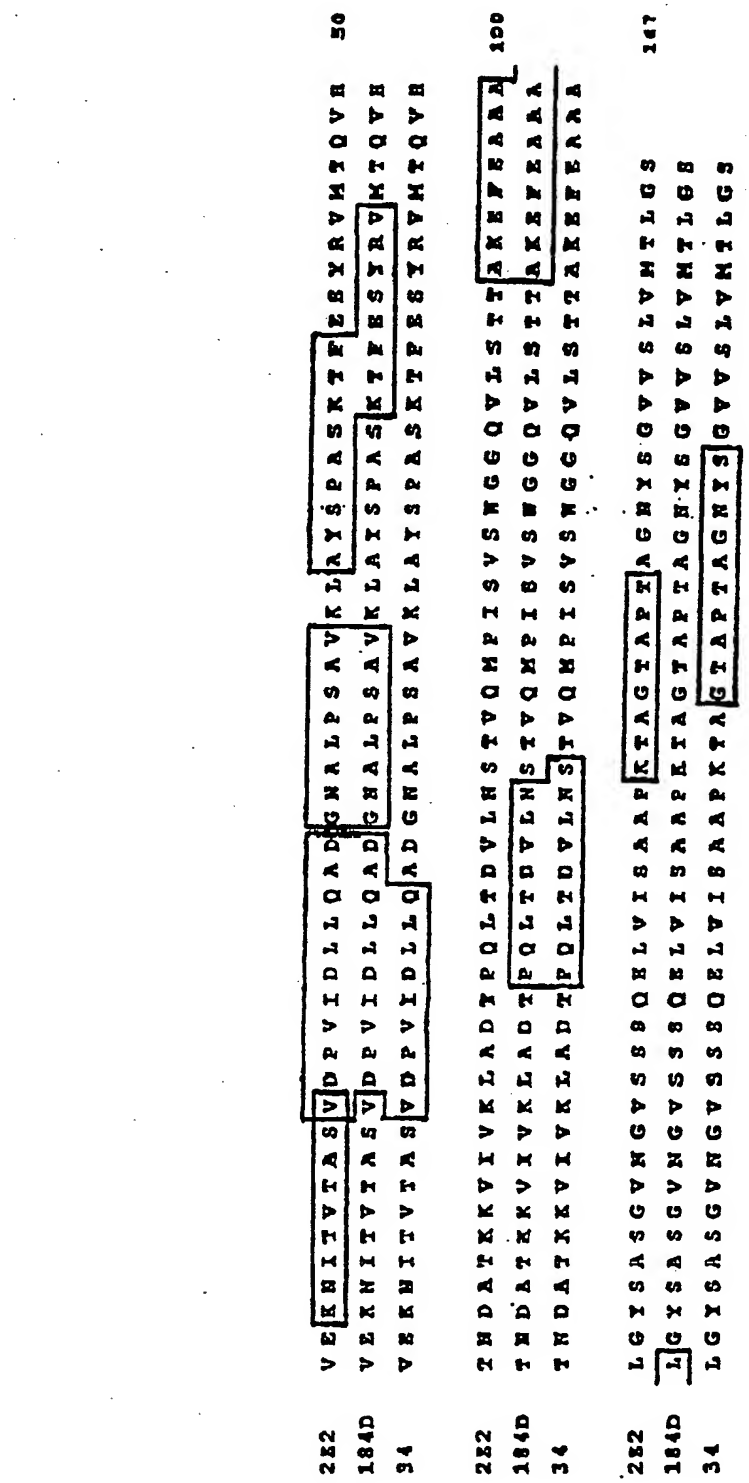


Figure 18

Monkey 184D(#1)

PROLIFERATIVE RESPONSES TO CFA/I SYNTHETIC PEPTIDE

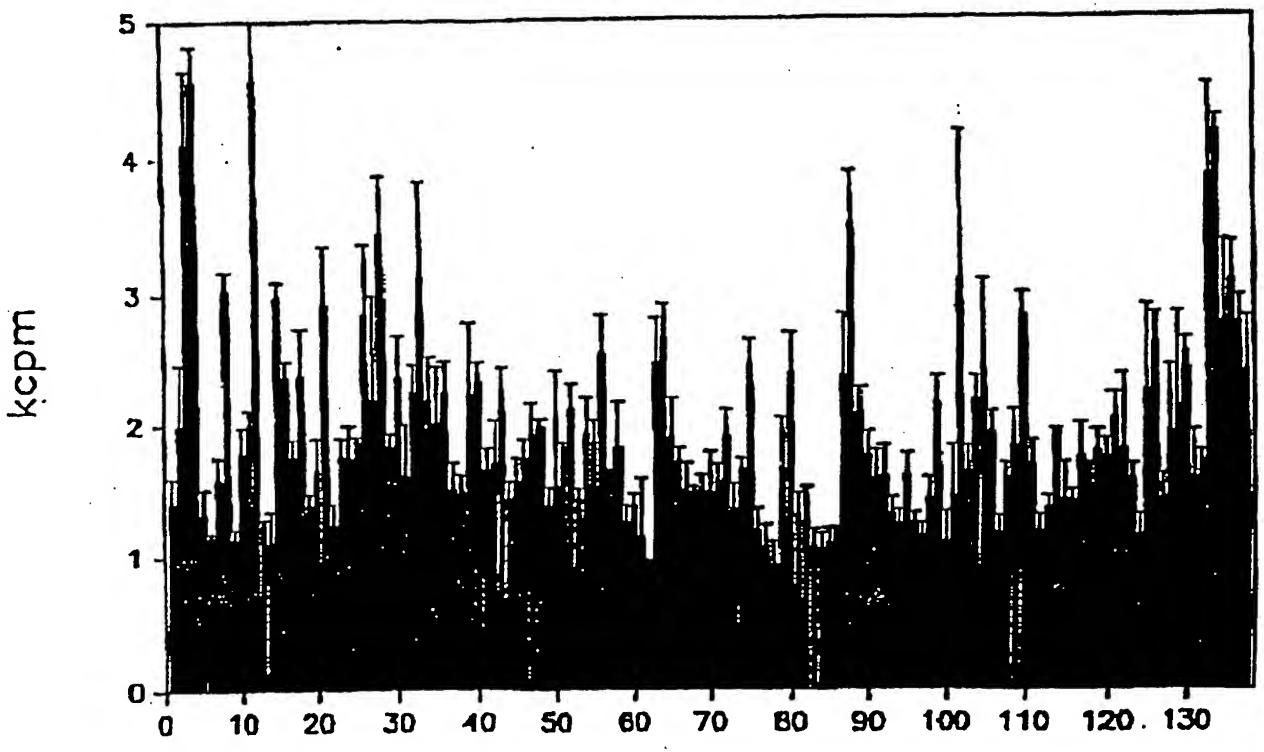


Figure 19

Monkey 3+ (#2)

PROLIFERATIVE RESPONSES TO CFA/I SYNTHETIC PEPTIDE

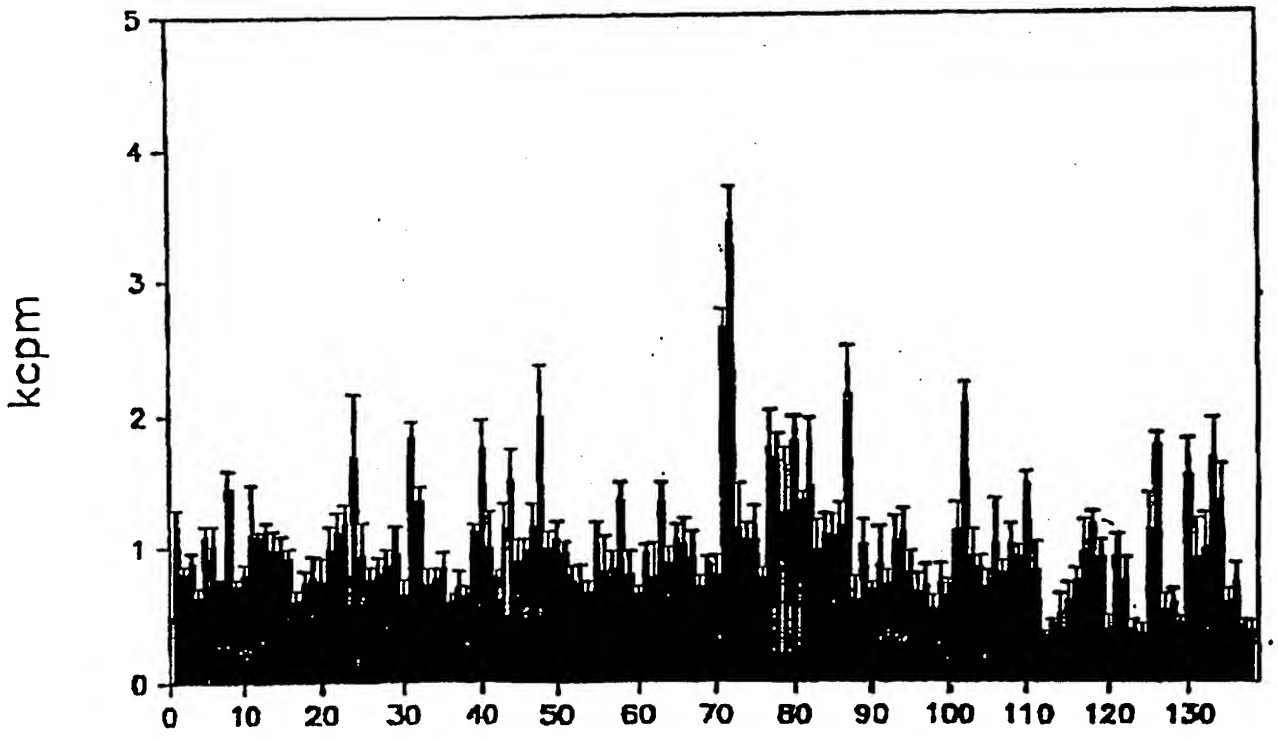


Figure 2a

Monkey 222 (#3)

PROLIFERATIVE RESPONSES TO CFA/I SYNTHETIC PEPTIDE

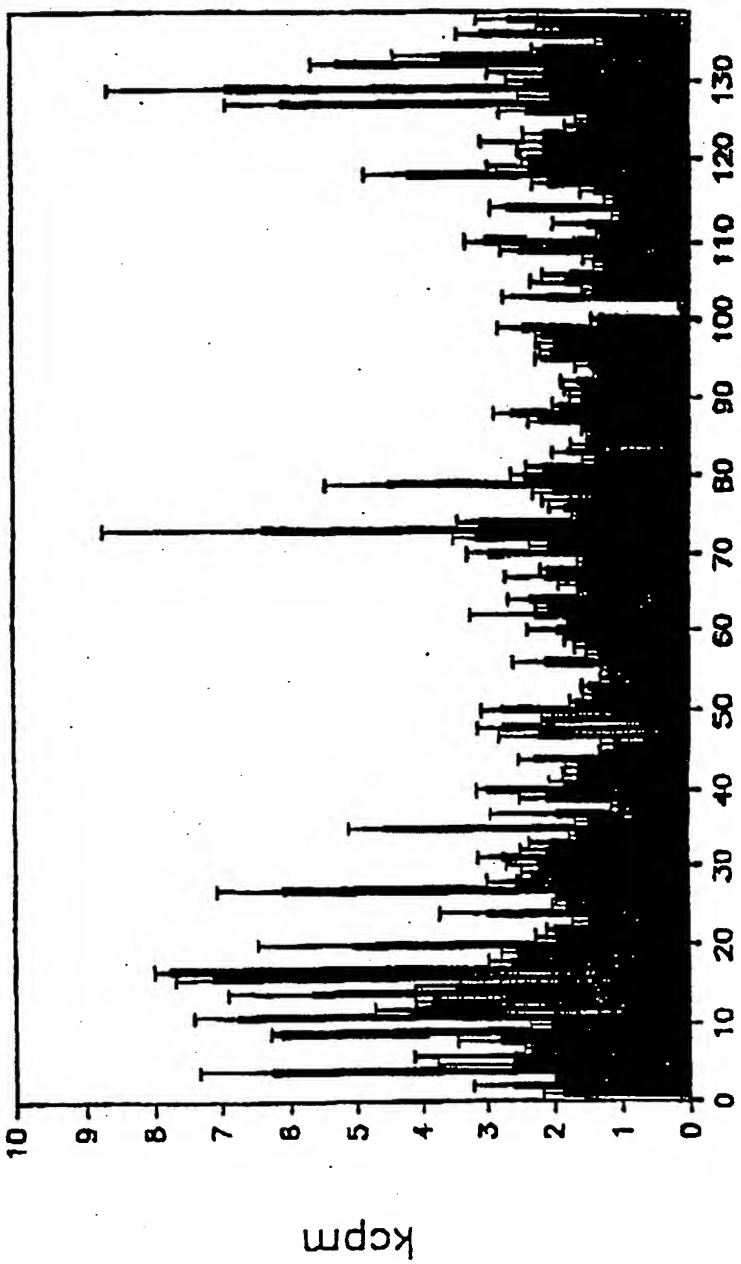


Figure #21

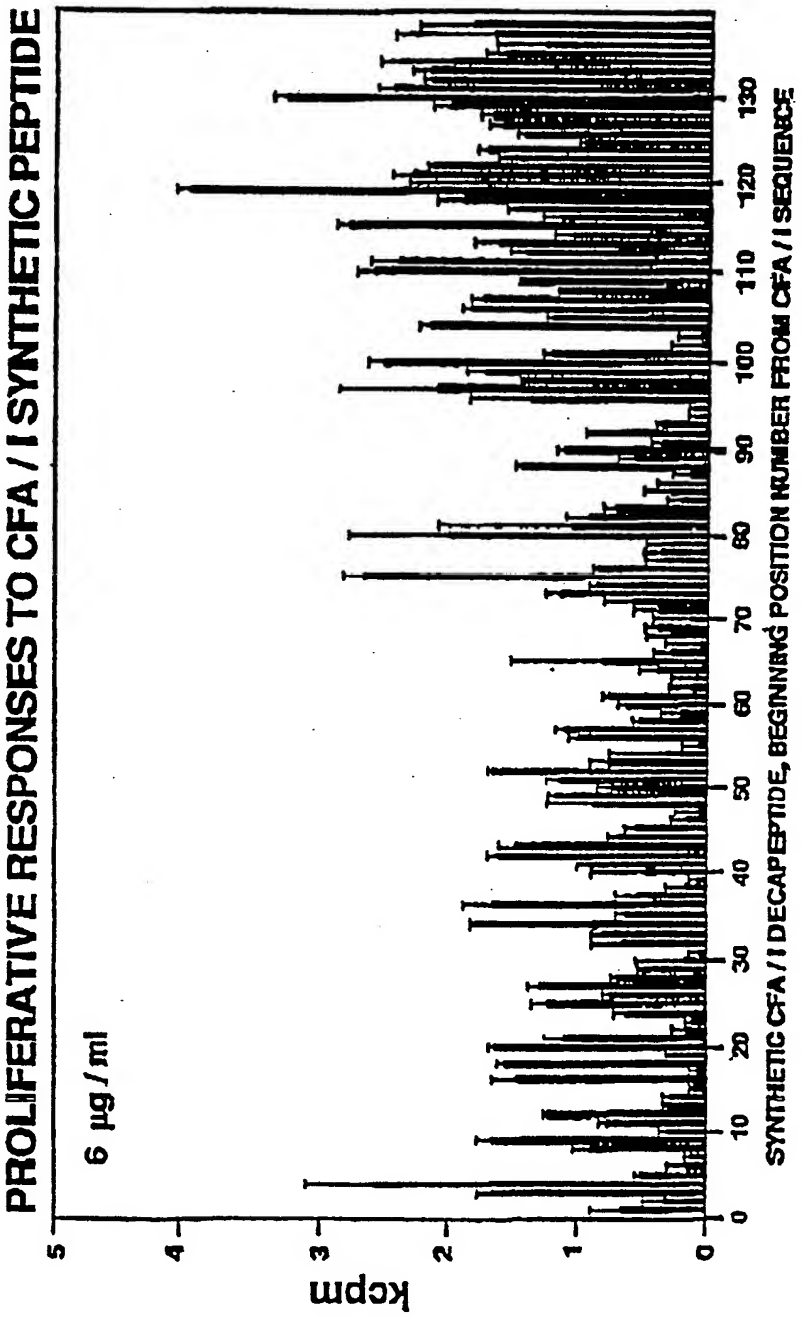

PROLIFERATIVE RESPONSES TO CFA / I SYNTHETIC PEPTIDE
6 µg / ml
kcpm
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
90
100
110
120
130
SYNTHETIC CFA / I DECAPEPTIDE, BEGINNING POSITION NUMBER FROM CFA / I SEQUENCE

Figure 22

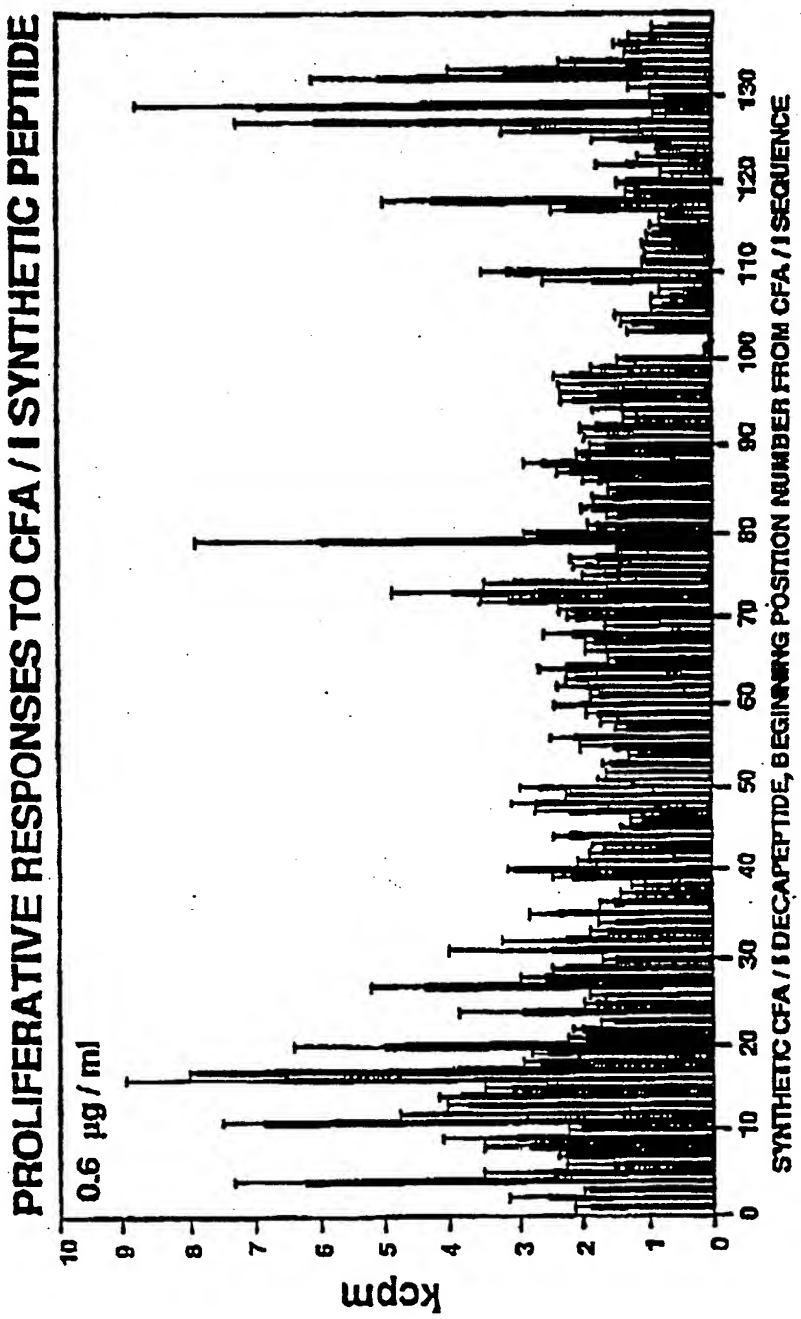

PROLIFERATIVE RESPONSES TO CFA / I SYNTHETIC PEPTIDE
0.6 µg / ml
kcpm
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60
70
80
90
100
110
120
130
SYNTHETIC CFA / I DECAPEPTIDE, BEGINNING POSITION NUMBER FROM CFA / I SEQUENCE

Figure #23

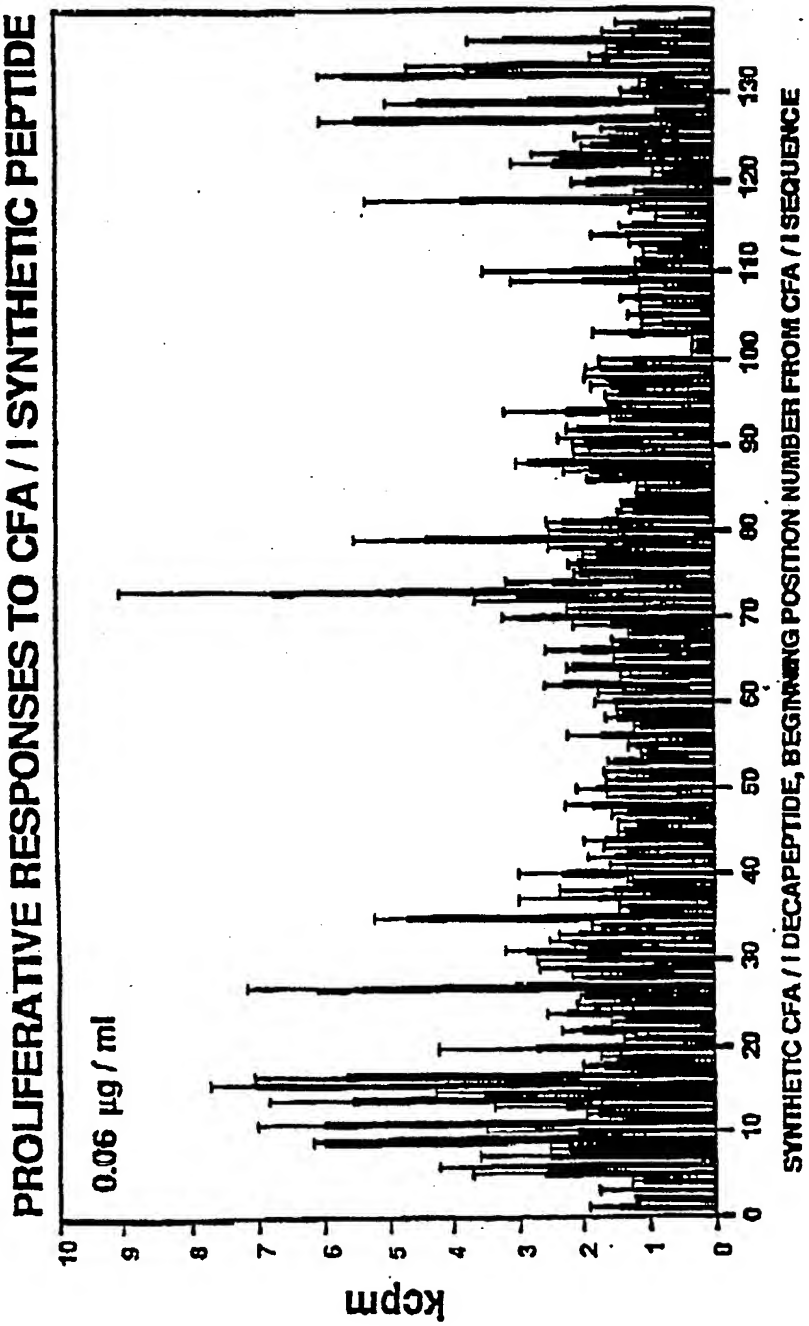

PROLIFERATIVE RESPONSES TO CFA / I SYNTHETIC PEPTIDE
0.06 µg / ml
kcpm
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60
70
80
90
100
110
120
130
SYNTHETIC CFA / I DECAPEPTIDE, BEGINNING POSITION NUMBER FROM CFA / I SEQUENCE

Figure 24

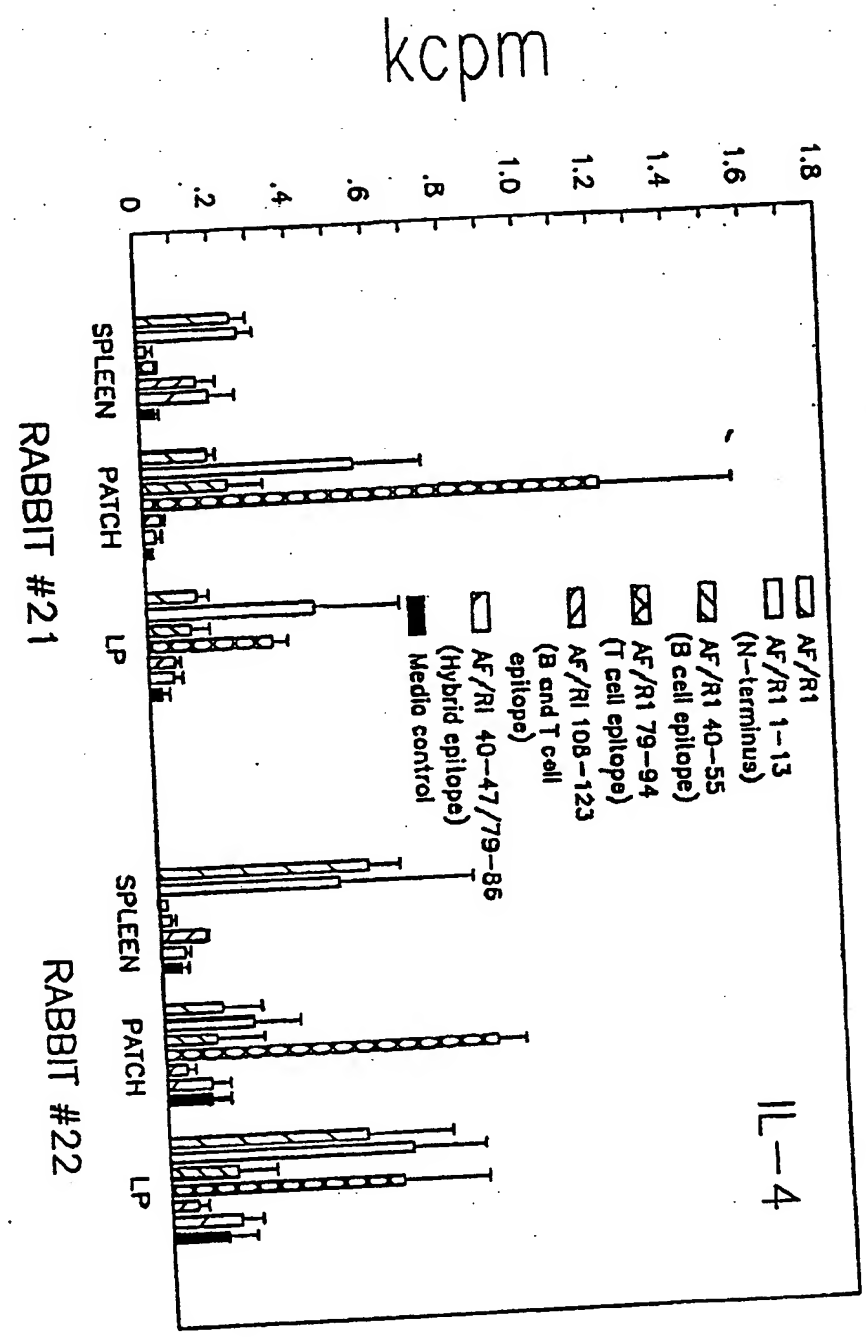

kcpm
RABBITS AT DAY 31 FOLLOWING ID IMMUNIZATION ON
DAYS 0, 7, 14, 21 WITH AF/RI IN MICROSPHERES
IL-4
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
AF/R1
AF/R1 1-13 (N-terminus)
AF/R1 40-55 (B cell epitope)
AF/R1 79-94 (T cell epitope)
AF/RI 108-123 (B and T cell epitope)
AF/RI 40-47/79-86 (Hybrid epitope)
Media control
SPLEEN
PATCH
LP
RABBIT #21
SPLEEN
PATCH
LP
RABBIT #22

Figure 25

RDEC-1 COLONIZATION

Immunized vs Unimmunized

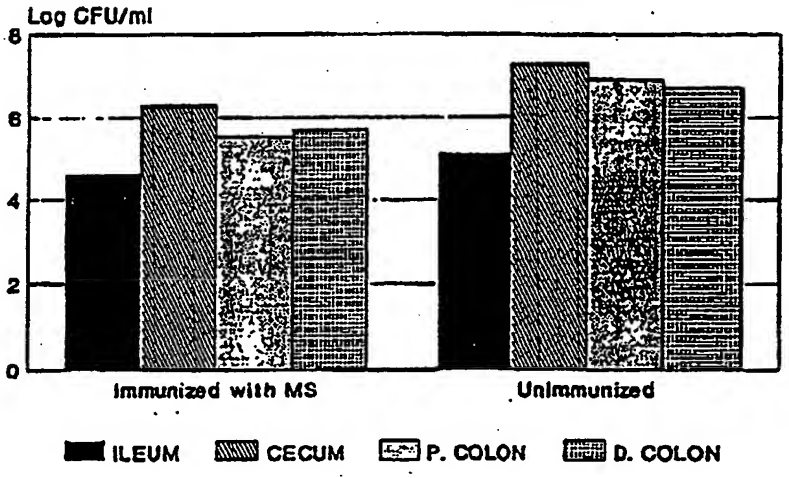
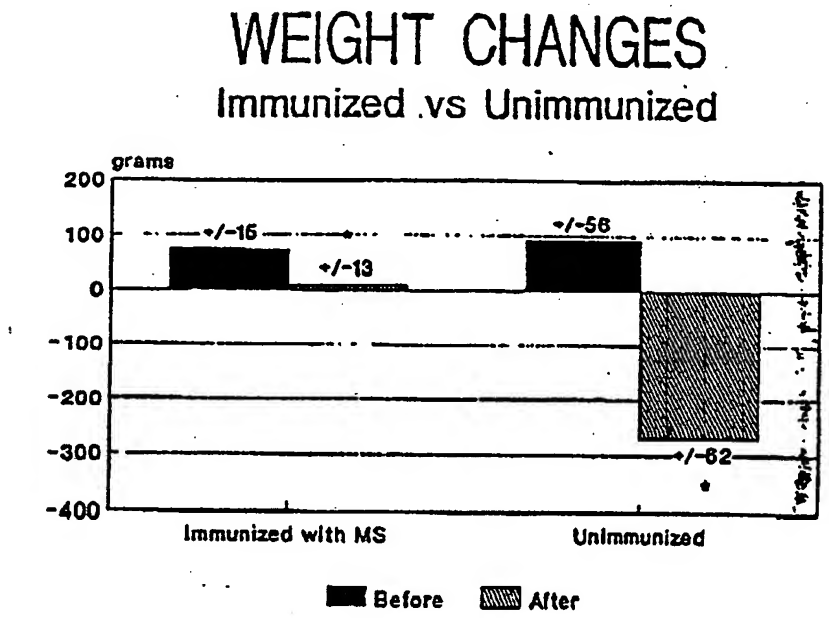


Figure 26

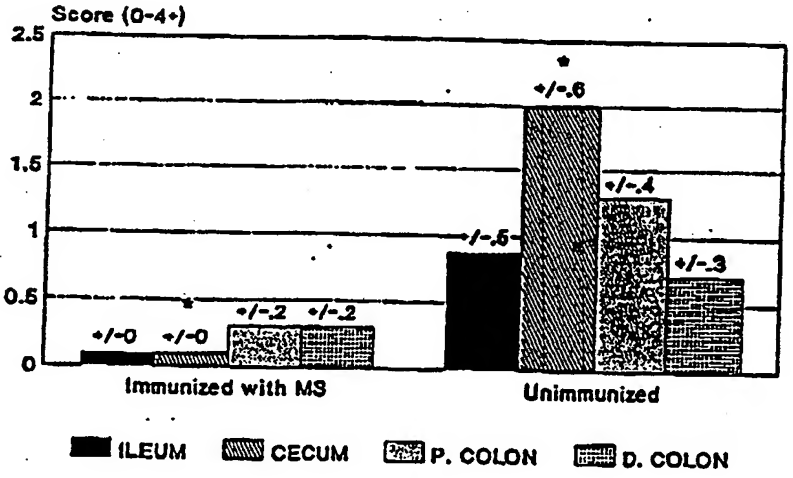


-P<.001

Figure 27

RDEC-1 ATTACHMENT

Immunized vs Unimmunized



* P<.01

Figure 28

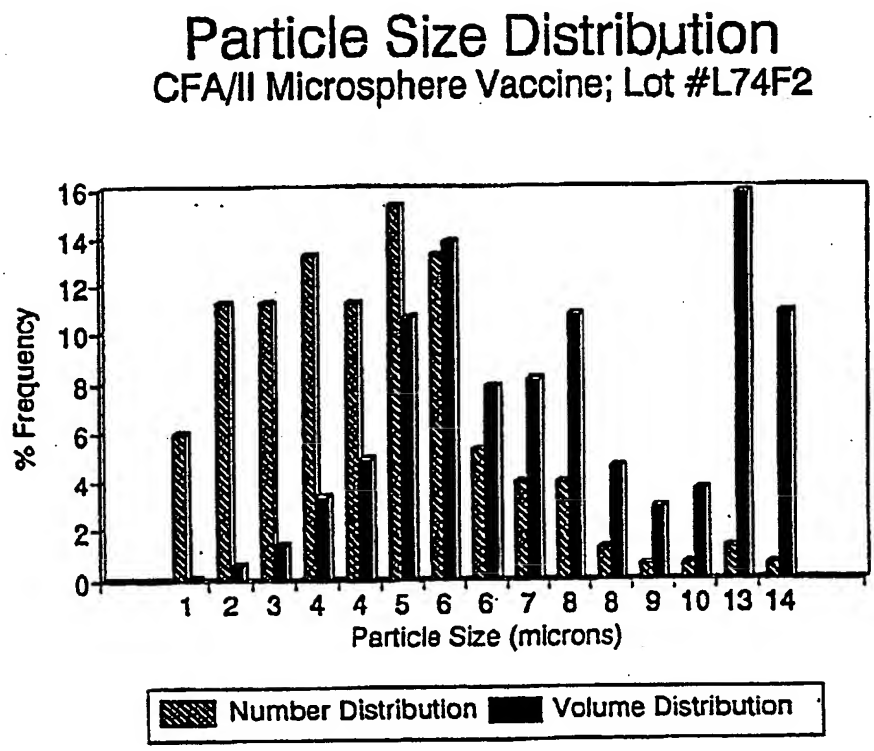

Particle Size Distribution
CFA/II Microsphere Vaccine; Lot #L74F2
% Frequency
0
2
4
6
8
10
12
14
16
1 2 3 4 4 5 6 6 7 8 8 9 10 13 14
Particle Size (microns)
Number Distribution
Volume Distribution

Figure 29

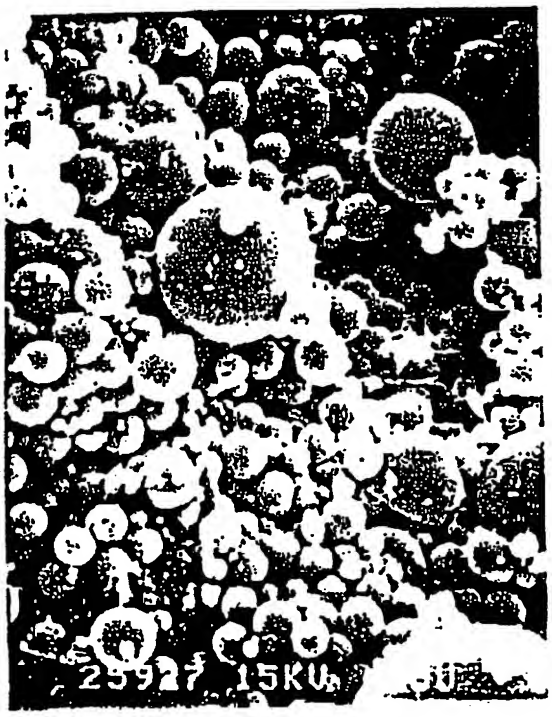


Figure 30

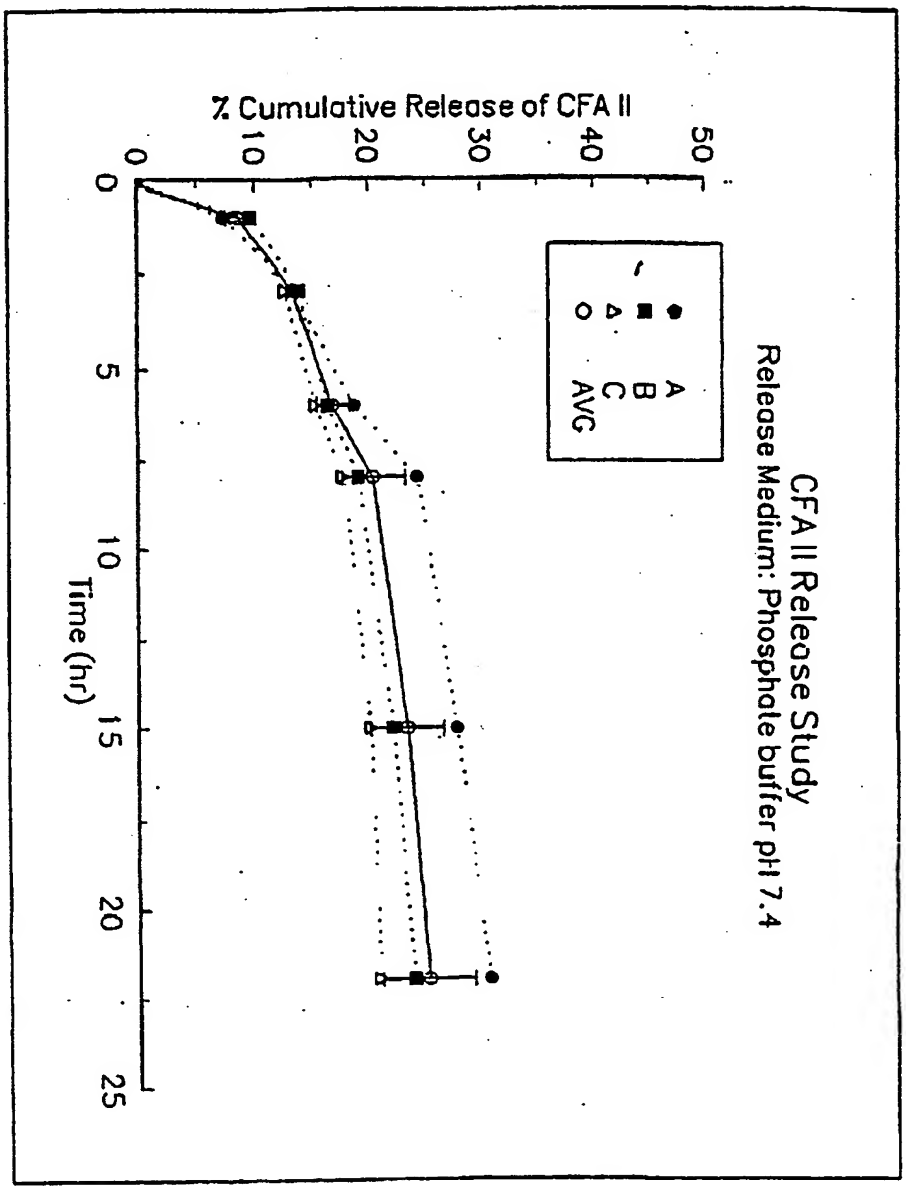

CFA II Release Study
Release Medium: Phosphate buffer pH 7.4
% Cumulative Release of CFA II
0
10
20
30
40
50
Time (hr)
0
5
10
15
20
25
A
B
C
AVG

Figure 31

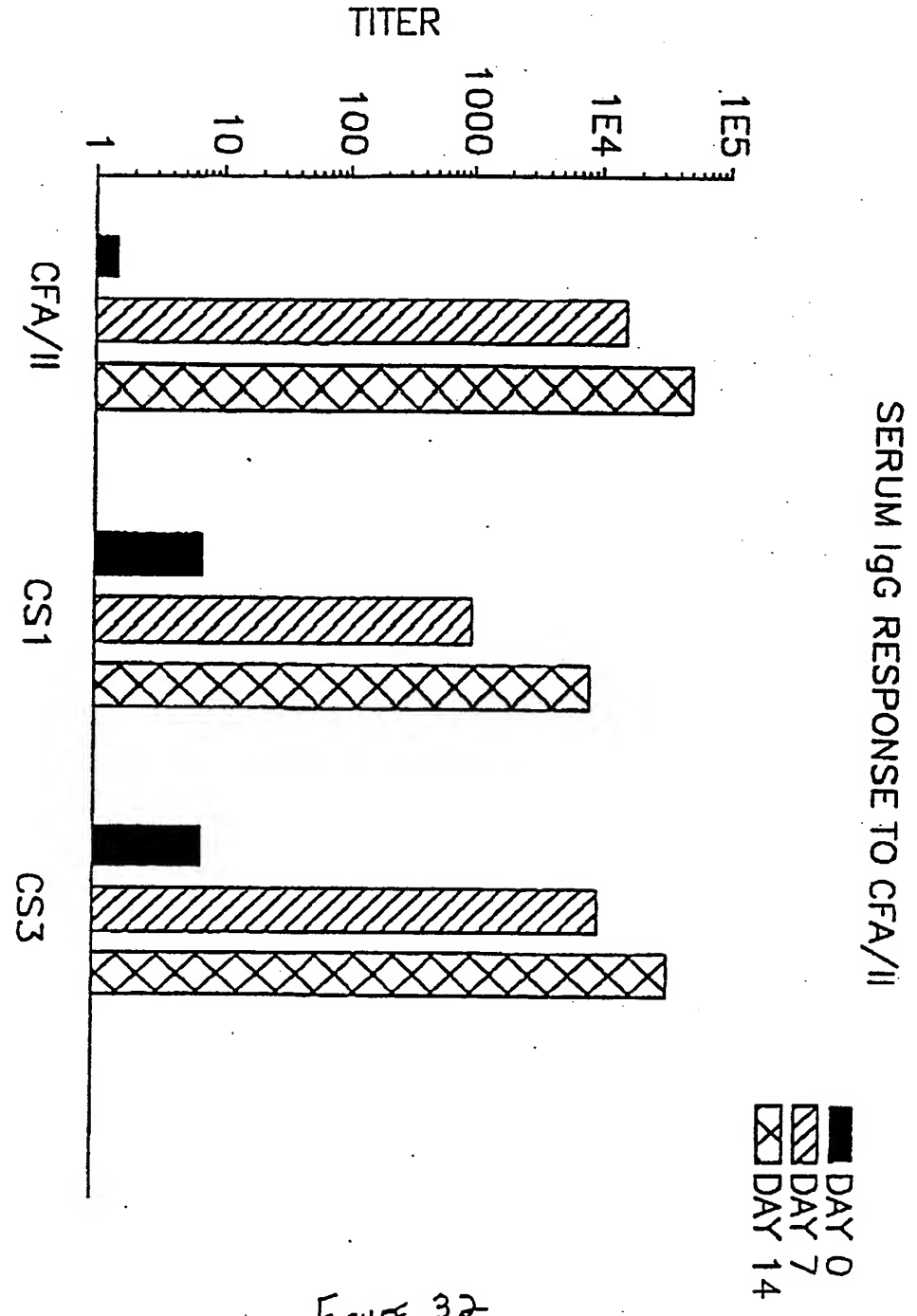

TITER
1
10
100
1000
1E4
1E5
CFA/II
CS1
CS3
SERUM IgG RESPONSE TO CFA/II
DAY 0
DAY 7
DAY 14

Figure 32

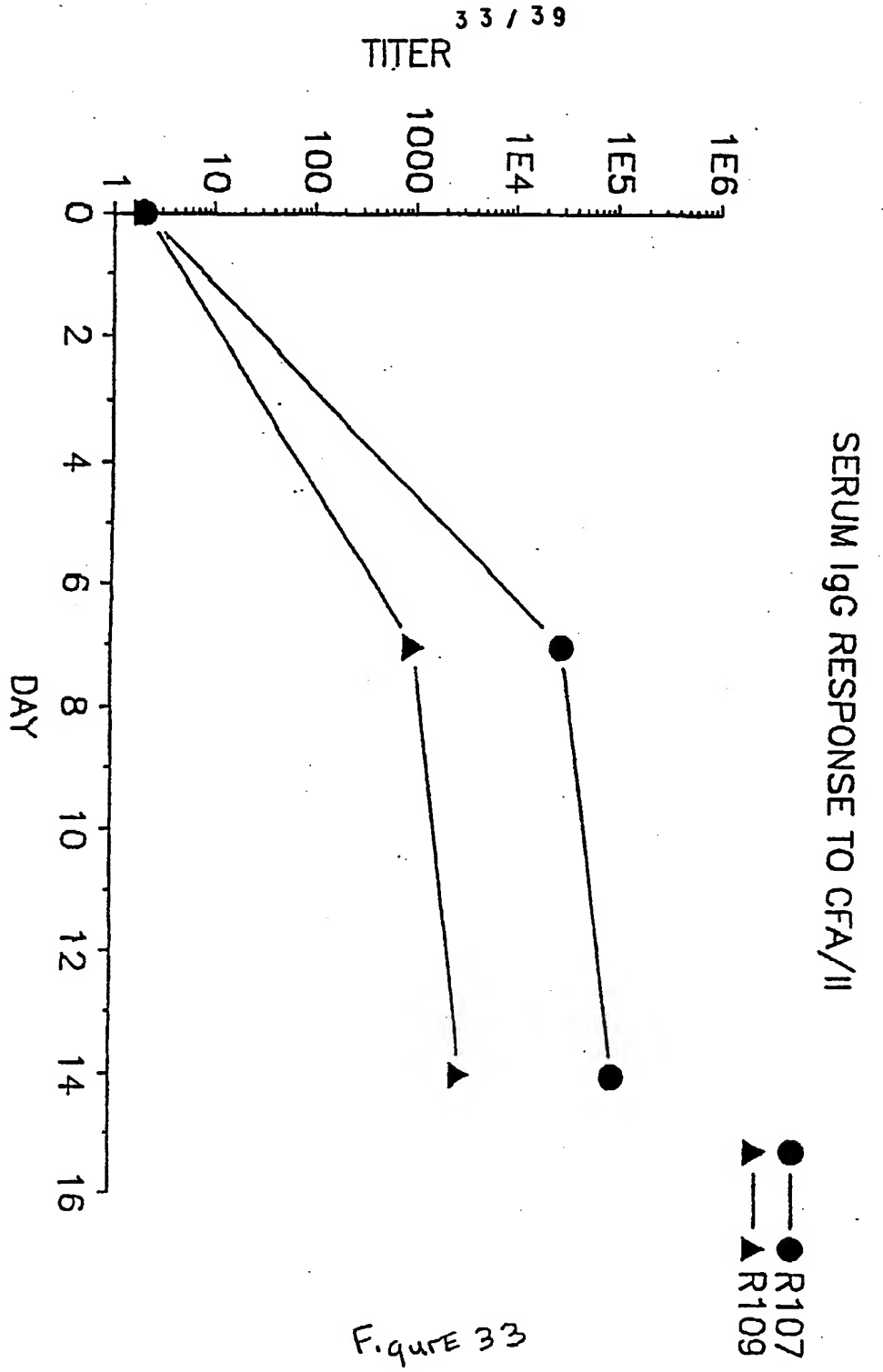

SERUM IgG RESPONSE TO CFA/II
TITER
1
10
100
1000
1E4
1E5
1E6
DAY
0
2
4
6
8
10
12
14
16
R107
R109

Figure 33

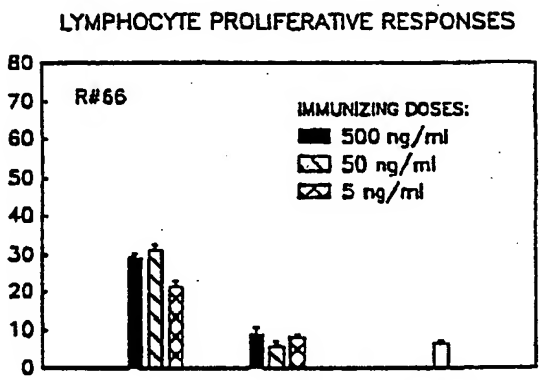


Figure (b)

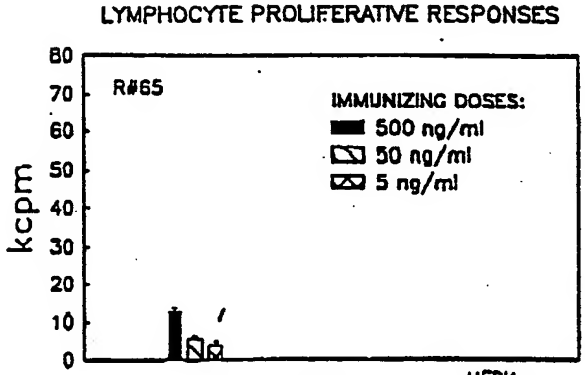


Figure (a)

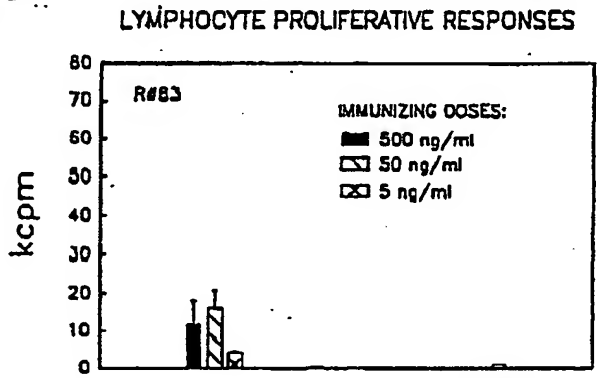


Figure (c)

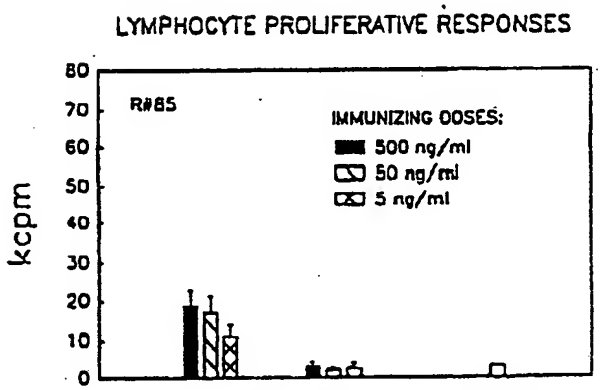
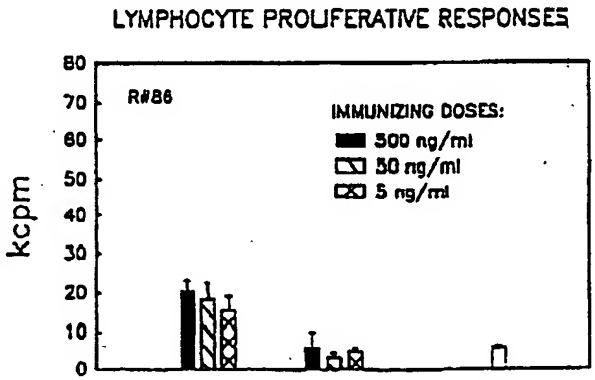


Figure (d)



(e)

Figure 34

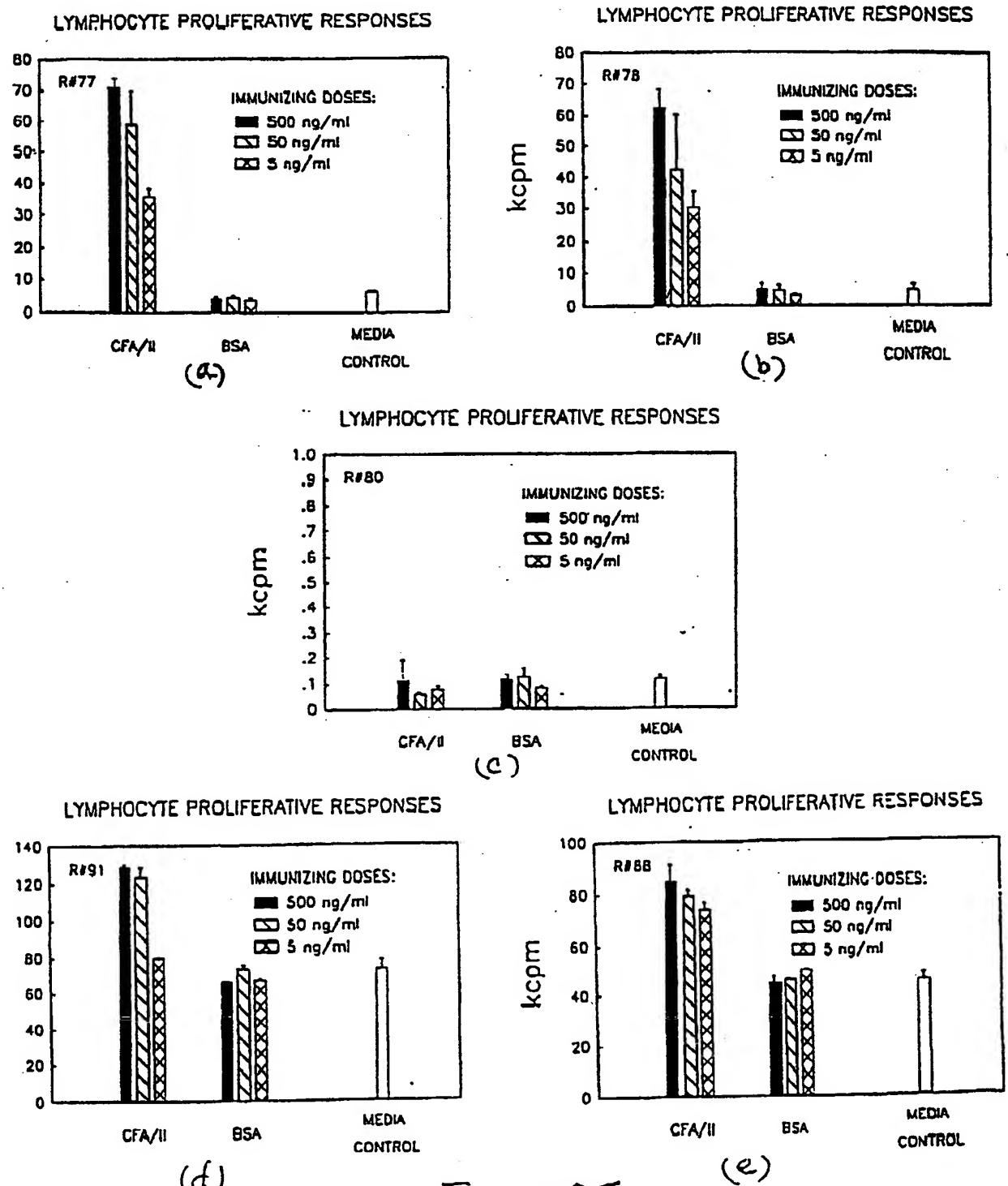
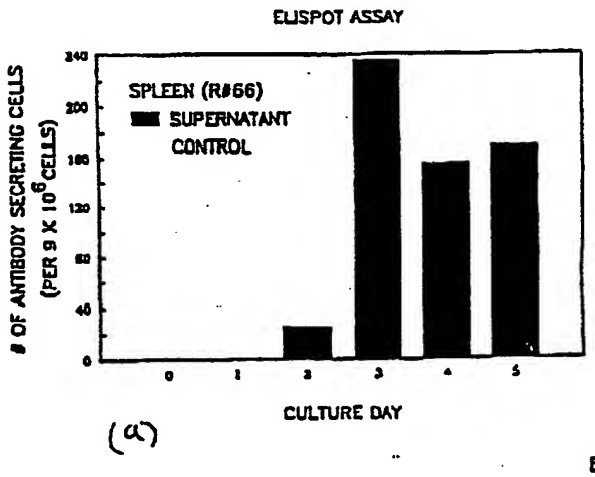
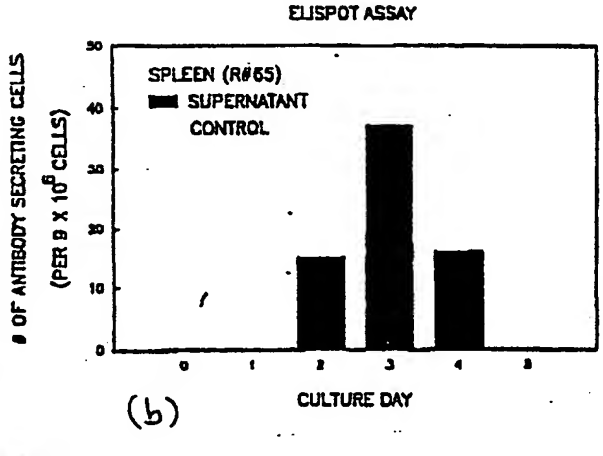

LYMPHOCYTE PROLIFERATIVE RESPONSES
R#77
IMMUNIZING DOSES:
500 ng/ml
50 ng/ml
5 ng/ml
CFA/II
BSA
MEDIA CONTROL
(a)
LYMPHOCYTE PROLIFERATIVE RESPONSES
R#78
kcpm
IMMUNIZING DOSES:
500 ng/ml
50 ng/ml
5 ng/ml
CFA/II
BSA
MEDIA CONTROL
(b)
LYMPHOCYTE PROLIFERATIVE RESPONSES
R#80
kcpm
IMMUNIZING DOSES:
500 ng/ml
50 ng/ml
5 ng/ml
CFA/II
BSA
MEDIA CONTROL
(c)
LYMPHOCYTE PROLIFERATIVE RESPONSES
R#91
IMMUNIZING DOSES:
500 ng/ml
50 ng/ml
5 ng/ml
CFA/II
BSA
MEDIA CONTROL
(d)
LYMPHOCYTE PROLIFERATIVE RESPONSES
R#88
kcpm
IMMUNIZING DOSES:
500 ng/ml
50 ng/ml
5 ng/ml
CFA/II
BSA
MEDIA CONTROL
(e)

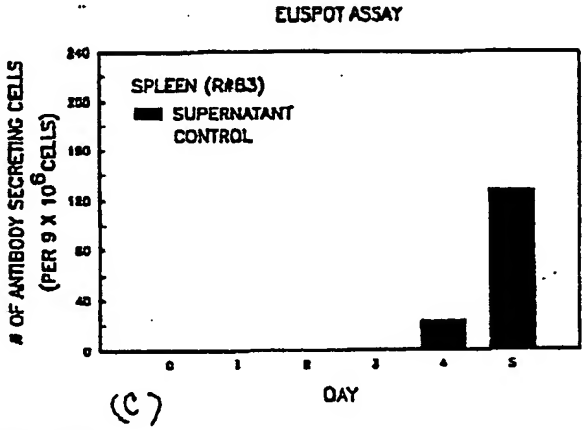
Figure 35


ELISPOT ASSAY
SPLEEN (R#66)
SUPERNATANT
CONTROL
OF ANTIBODY SECRETING CELLS
(PER 9 X 10^6 CELLS)
CULTURE DAY

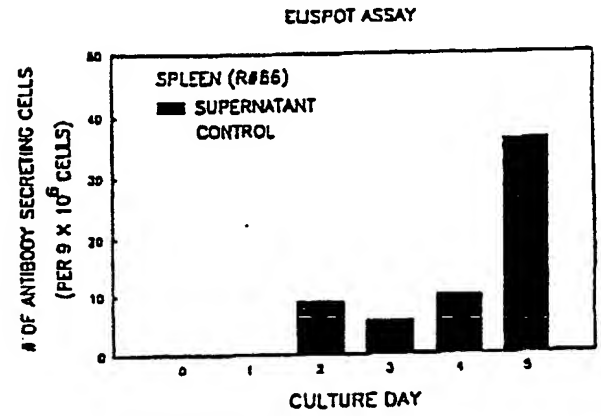
(a)


ELISPOT ASSAY
SPLEEN (R#65)
SUPERNATANT
CONTROL
OF ANTIBODY SECRETING CELLS
(PER 9 X 10^6 CELLS)
CULTURE DAY

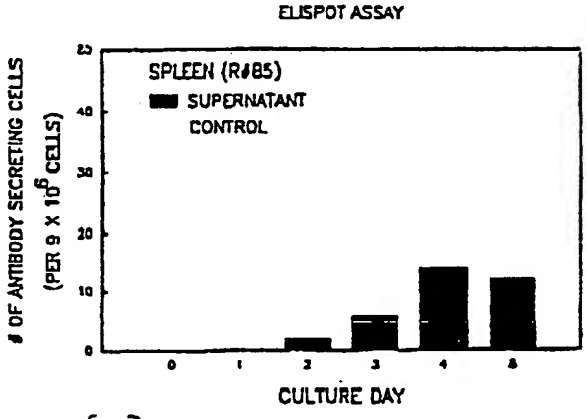
(b)


ELISPOT ASSAY
SPLEEN (R#63)
SUPERNATANT
CONTROL
OF ANTIBODY SECRETING CELLS
(PER 9 X 10^6 CELLS)
DAY

(c)


ELISPOT ASSAY
SPLEEN (R#66)
SUPERNATANT
CONTROL
OF ANTIBODY SECRETING CELLS
(PER 9 X 10^6 CELLS)
CULTURE DAY

(d)


ELISPOT ASSAY
SPLEEN (R#85)
SUPERNATANT
CONTROL
OF ANTIBODY SECRETING CELLS
(PER 9 X 10^6 CELLS)
CULTURE DAY

(e)

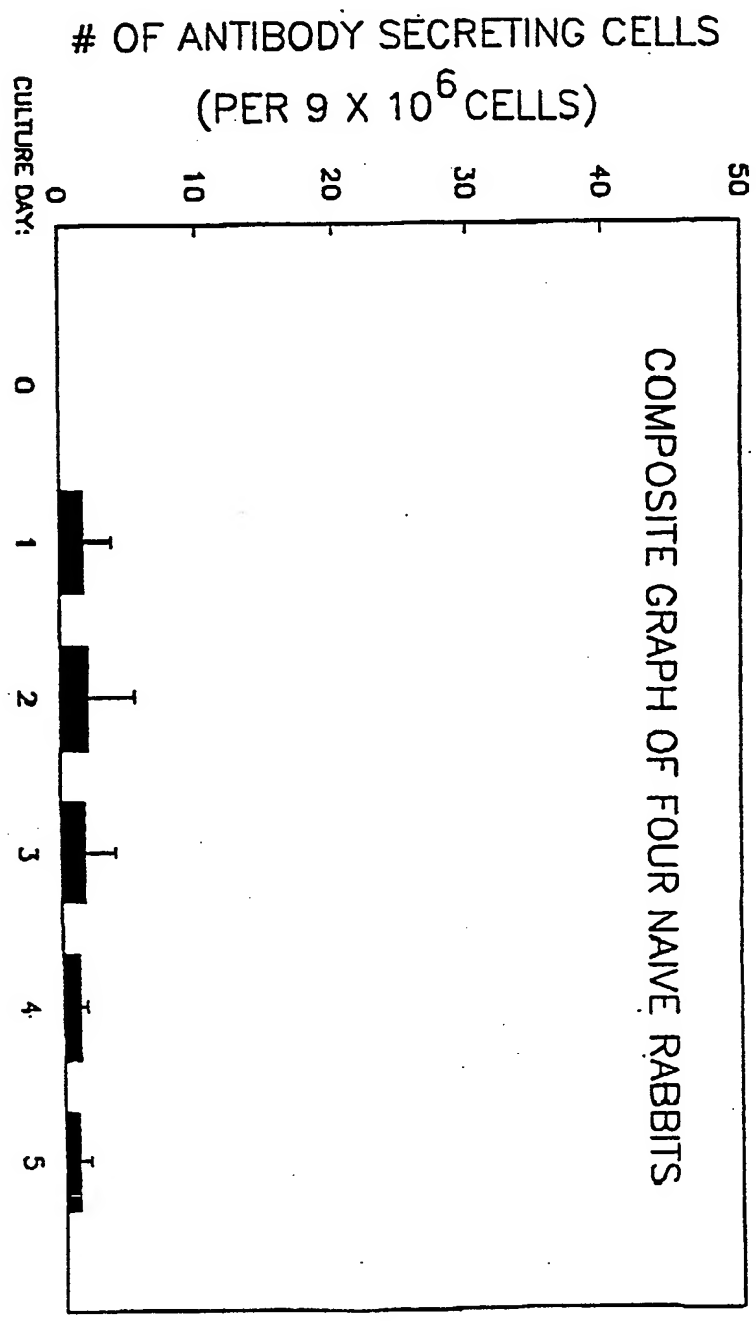

OF ANTIBODY SECRETING CELLS
(PER 9 X 10^6 CELLS)
CULTURE DAY:
0
10
20
30
40
50
0
1
2
3
4
5
COMPOSITE GRAPH OF FOUR NAIVE RABBITS
ELISPOT ASSAY

Figure 37

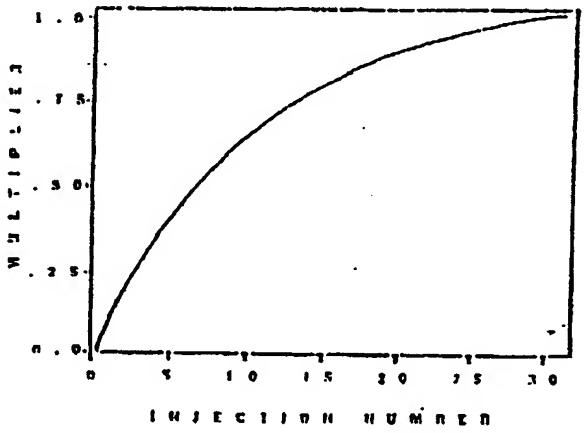


Figure 38

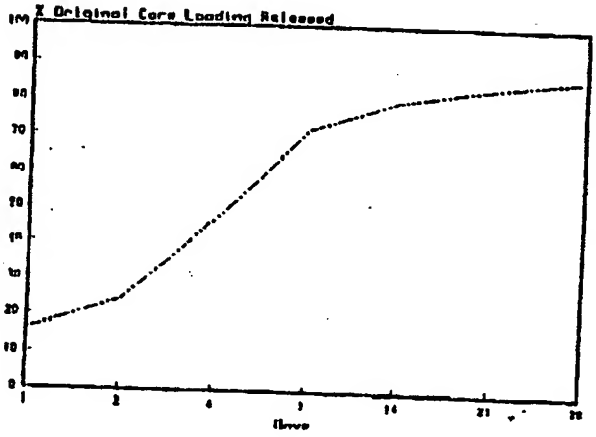

% Original Core Loading Released
100
90
80
70
60
50
40
30
20
10
0
1
2
4
7
14
21
28
Time

Figure 39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/02536

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/02, 9/26
US CL : 424/85, 88, 89, 92, 417, 422, 450, 458, 469
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85, 88, 89, 92, 417, 422, 450, 458, 469

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
GLUCOID (P) LACTIDE (P) ORAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,897,268 (TICE ET AL.) 30 JANUARY 1990, COLUMN 2, LINE 6 TO COLUMN 3, LINE 25.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 JUNE 1994	JUN 23 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer THEODORE J. CRIARES Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*